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RESEARCH ARTICLE

Influenza virus replication is affected by glutaredoxin1-mediated protein deglutathionylation

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Abstract

Several redox modifications have been described during viral infection, including influenza virus infection, but little is known about glutathionylation and this respiratory virus. Glutathionylation is a reversible, post-translational modification, in which protein cysteine forms transient disulfides with glutathione (GSH), catalyzed by cellular oxidoreductases and in particular by glutaredoxin (Grx). We show here that (i) influenza virus infection induces protein glutathionylation, including that of viral proteins such as hemagglutinin (HA); (ii) Grx1-mediated

Abbreviations: 2-AAPA, R,R′-2-Acetylamino-3-[4-(2-acetylamino-2carboxyethylsulfanylthiocarbonylamino) phenylthiocarbamoylsulfanyl] propionic acid hydrate; BioGEE, glutathione ethyl ester biotin amide; Cys, cysteine; DAPI, 4′6-diamidino-2-phenylindole; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gp120, glycoprotein 120; Grxs/Grx1, glutaredoxins/glutaredoxin1; GSH, glutathione; H_2O_2, hydrogen peroxide; HA, hemagglutinin; HAU, hemagglutinating unit; HCV, hepatitis C virus; HED, 2-hydroxyethyl disulfide; HIV, human immunodeficiency virus; HPLC, high-performance liquid chromatography; HSV, herpes simplex virus; IL-1β, interleukin-1β; IL-6, interleukin-6; m.o.i., multiplicity of infection; M1/M2, matrix protein 1/2; MDCK, madin-darby canine kidney; NA, neuraminidase; NADPH, nicotinamide adenine dinucleotide phosphate; NEM, N-ethylmaleimide; NP, nucleoprotein; NS5, nonstructural protein 5; nsP2, nonstructural protein2; NWS, influenza A/NWS/33 H1N1; PBS, phosphate-buffered saline; pH1N1, pandemic A/California/04/09 H1N1; PR8, influenza A/Puerto Rico/8/34 H1N1; PSSG, glutathionylated proteins; RNS, reactive nitrogen species; ROS, reactive oxygen species; RPMI 1640, Roswell park memorial institute 1640; RT- qPCR, reverse transcription-quantitative polymerase chain reaction; SARS-CoV-2, severe acute respiratory syndrome-coronavirus-2; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; siRNA, small interfering RNA; Strep-POD, streptavidin-peroxidase; TCID50, tissue culture infectious dose 50%; TNFα, tumor necrosis factorα; Trx, Thioredoxin; WB, western blot.

Lucia Nencioni and Anna Teresa Palamara contributed equally to this study.

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deglutathionylation is important for the viral life cycle, as its inhibition, either with an inhibitor of its enzymatic activity or by siRNA, decreases viral replication. Overall these data contribute to the characterization of the complex picture of redox regulation of the influenza virus replication cycle and could help to identify new targets to control respiratory viral infection.

KEYWORDS

deglutathionylation, glutaredoxin1, glutathione, glutathionylation, influenza virus, redox-regulation

1 | INTRODUCTION

Redox-dependent changes are implicated in different aspects of viral infections, at the level of viral replication[1,2] and pathogenesis, particularly of respiratory viruses.[3–7]

It is now appreciated that oxidative stress, historically defined as an imbalance between the production of oxidant species, mainly reactive oxygen and nitrogen species (ROS, RNS), and antioxidant systems of the cell, leads to disruption of redox signaling.[8] Several groups, including ours, have reported overproduction of ROS and impairment of antioxidant defenses, such as reduced glutathione (GSH) levels, during influenza virus infection; of note, perturbation of redox-sensitive pathways, including the immune ones, favors influenza virus replication.[9–13] In particular, depletion of GSH affects the folding and maturation of the viral glycoprotein hemagglutinin (HA) and the progression of the virus life-cycle.[9]

Importantly, GSH is not only an antioxidant molecule but is fundamental in the redox regulation of several cellular functions.[14–16] In addition to determine the H_2O_2 level, that acts as an intracellular messenger in signal transduction, GSH can play a direct role in cell signaling through the formation of mixed disulfides with proteins, a process known as glutathionylation, that can be regulated in infections, immune and inflammatory responses.[17,18] In fact, protein glutathionylation meets two fundamental requirements for a regulatory role, reversibility and enzymatic catalysis; in particular, the reaction can be reversibly catalyzed by thiol-disulfide oxidoreductases, such as glutaredoxins (Grxs).[19] Glutathionylation of cellular proteins has been observed in infection models with different viruses, including parainfluenza virus,[20] HIV,[21] HSV,[22] and more recently, SARS-CoV-2.[23] Other studies have demonstrated that some viral proteins can undergo glutathionylation, resulting in different effects on their functions; examples include retroviral protease, chikungunya virus nsP2, dengue and zika NS5, HCV polymerase and SARS-CoV-2 protease.[24–28]

Protein deglutathionylation is mainly catalyzed by Grxs, enzymes belonging to the Thioredoxin (Trx) fold family, with whom they share a well-conserved cysteine-containing structural motif important for their oxidoreductase activity.[19] So far, four Grxs have been identified in mammals (1–3, 5, and); Grx1 has a dithiol active site (Cys-X-X-Cys) and is mainly localized in the cytosol. Bacterial Grxs, and a Grx homolog in viruses, have also been identified[19]; in particular, vaccinia virus O2L open reading frame encodes for a functional enzyme with thioltransferase activity[29] and an enzyme regulating the activity of glutathionylated HIV protease has been detected within HIV virions.[30]

While several redox modifications have been described during influenza virus infection, little is known about glutathionylation and, to our knowledge, there are no data on Grx1 and the influenza virus.

The aim of the present study was to analyze the glutathionylation process during influenza virus infection and investigate the possible role of Grx1 in the regulation of viral replication. For this purpose, we used different models of infections: canine kidney epithelial cells MDCK and human lung epithelial cells A549 infected with influenza A virus A/Puerto Rico/8/34 H1N1 (PR8) and the murine macrophage cell line RAW264.7 infected with influenza A virus strains PR8, A/NWS/33 H1N1 (NWS), and pandemic A/California/04/09 H1N1 (pH1N1). We studied Grx1 levels and activity in infected epithelial cells and the effects of Grx1 inhibition on viral replication; to inhibit Grx1 we used a chemical inhibitor or short interfering RNA (siRNA).

The results indicate that protein glutathionylation, including of viral proteins, is induced by the influenza virus, and that Grx1 deglutathionylation activity is important for the virus to complete its replication cycle.

2 | MATERIALS AND METHODS

2.1 | Cell culture

Madin-Darby canine kidney (MDCK) cells and A549 human lung carcinoma cells were grown in RPMI 1640 and DMEM medium respectively, supplemented with 10% fetal bovine serum (FBS), 0.3 mg/ml glutamine,

100 U/ml penicillin, and 100 μg/ml streptomycin. RAW264.7 mouse macrophage cells were grown as described for A549 cells.

2.2 | Viruses infection and titration

Influenza A virus strains A/Puerto Rico/8/34 H1N1 (PR8), A/NWS/33 H1N1 (NWS), and pandemic A/California/04/09 H1N1 (pH1N1) were grown in allantoic cavities of 10 days old embryonated chicken eggs and harvested after 48 h at 37°C. To perform a single- or multi-cycle of infection, epithelial cells were challenged with PR8 at a multiplicity of infection (m.o.i.) of 1 or 0.1 for 1 h at 37°C, washed with PBS and incubated with medium supplemented with 2% FBS for 8 h or 24 h, respectively. Mouse macrophages were challenged with NWS, pH1N1, or PR8 at 10, 1, and 0.1 m.o.i. Virus production was determined in the supernatants of infected cells by measuring the hemagglutinating unit (HAU) or the tissue culture infectious dose 50 (TCID50), as previously described.[31]

2.3 | Cell treatments

Glutathione ethyl ester biotin amide (BioGEE, Invitrogen) was dissolved in DMSO and then diluted to a final concentration of 200 μM in a cell-culture medium.[32] N-ethylmaleimide (NEM, Sigma-Aldrich) was added to a final concentration of 40 mM for cell lysis to avoid thiol-disulfide exchange.

R,R′-2-Acetylamino-3-[4-(2-acetylamino-2carboxy ethylsulfanylthiocarbonylamino) phenylthiocarbamoylsulfanyl] propionic acid hydrate (2-AAPA, Sigma-Aldrich) was dissolved in DMSO and then diluted to the final concentrations in a cell-culture medium; its cytotoxicity was evaluated on A549 cells by treating the cells with concentration from 25 to 200 μM for 20 min, as in Sadhu et al.[33]; then the medium was changed and left for the following 24 h, after which cells were detached and counted by Trypan Blue staining. 2-AAPA was added to A549 cells at a concentration of 100 μM, 20 min before viral infection.

2.4 | GSH and PSSG assay

Intracellular GSH was assayed upon the formation of S-carboxymethyl derivatives of the free thiol with iodoacetic acid, followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives by the reaction with 1-fluoro-2,4-dinitrobenzene and quantified through high-performance liquid chromatography (HPLC). Mixed disulfides were determined by HPLC, after the reduction of cell lysates with $NaBH_4$ for 30 min at 40°C, as described in Ciriolo et al.[20]

2.5 | Western blot and immunoprecipitation

Cells were lysed with lysis buffer supplemented with NEM, phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Sigma-Aldrich) for 30 min on ice. Protein concentration was determined with DC Protein Assay (Bio-Rad). Then cell lysates were analyzed by SDS-PAGE under reducing or non-reducing conditions (i.e., without treatment with DTT) followed by Western blot (WB). Biotinylated proteins were visualized using Streptavidin-peroxidase (POD) conjugated (Roche) at 1:20 000 dilution. The other proteins were visualized using the following primary and secondary horseradish peroxidase (HRP)-conjugated antibodies: anti-Influenza (Merck Millipore), anti-M2, anti-Grx1, anti-ATF6, anti-IRE1 and anti-GAPDH (Santa Cruz Biotechnology), anti-p-IRE1 (Novus Biologicals), anti-Actin (Sigma Aldrich); anti-goat, anti-rabbit, and anti-mouse (Bethyl Laboratories). Membranes were developed with the WesternBright ECL HRP substrate (Advansta); when indicated, densitometric analysis was performed using ImageJ.

For immunoprecipitation, cell lysates from BioGEE-treated and virus-infected cells, blocked with NEM to prevent the artifactual formation of disulfides, were incubated with anti-influenza antibody at 1:100 dilution at 4°C for 4 h, then the immunocomplexes were precipitated with Protein A/G Plus-Agarose (Santa Cruz Biotechnology) at 4°C overnight. After washing with cold PBS, agarose beads were eluted by boiling them in a sample buffer for 5 min. Eluate was split into two aliquots, one left untreated and one reduced with 10 mM DTT for 5 min, loaded on gels, and analyzed by WB with streptavidin-POD as described above. After stripping, the membranes were reprobed with an anti-HA antibody (Santa Cruz Biotechnology).

2.6 | RT-qPCR

Total RNA was extracted from cells with TRIzol (Sigma-Aldrich) and RNA quality and concentration were measured with a NanoDrop spectrophotometer. Reverse-transcription (RT) and quantitative PCR (qPCR) were performed using SensiFAST cDNA Synthesis kit (Bioline) for viral HA, M2 and human GRX1, and actin was used as a

reference gene for normalization. The primer sequences used were the following:

HA	F: ATTCCGTCCATTCAATCCAGAGG
	R: ACCATCCATCTATCATTCCAGTCC
M2	F: GCAAGCGATGAGAACCATTGG
	R: GCGGCAATAGCGAGAGGATC
GRX1	F: GGAGCAAGAACGGTGCCTCGAG
	R: AAAGCAGATTGGAGCTCTGCAG
ACTIN	F: AGCGTCTTGTCATTGGCGAA
	R: TTTTCTGCTCCAGGCGGACT

Relative quantitative evaluation was performed by the comparative ΔΔCt method.[12]

Viral RNA in supernatants was quantified using a standard curve consisting of serial dilutions of a plasmid containing the PR8 genome with a known titer (range, 10^5 cp/ml–10^2 cp/ml). The amount of cellular RNA was quantified simultaneously using a SYBR GREEN PCR for the housekeeping β-globin gene and used to normalize the PR8 RNA.

2.7 | Grx1 deglutathionylation activity (HED assay)

In this assay, first an artificial substrate, 2-hydroxyethyl disulfide (HED), reacts with GSH forming mixed disulfides, then Grx1 makes a nucleophilic attack and releases the deglutathionylated substrate. Briefly, cells lysed with lysis buffer (100 mM Tris–HCl, 150 mM NaCl, 1% NP-40) at pH 7.9, cleared by centrifugation, equalized for protein content were incubated with reaction buffer consisting of 1 mM GSH, 0.2 mM NADPH, 2 mM EDTA, 1.2 U glutathione reductase, in 100 mM Tris–HCl pH 7.9. To 500 µl of this mixture, HED (Sigma-Aldrich) was added to a final concentration of 0.7 mM. The decrease in absorbance (indicative of the consumption of NADPH) was followed spectrophotometrically at 340 nm. Grx1 activity was determined after subtracting the spontaneous reduction rate observed in the absence of cell lysates and was expressed as micromoles of NADPH oxidized/min/mg protein.[34]

2.8 | Grx1 silencing

Semi-confluent monolayers of A549 cells were transfected with Grx1 siRNA (or control siRNA, Santa Cruz Biotechnology) following preincubation with Lullaby transfection reagent (Oz Biosciences), according to the manufacturer's instructions, for 24 h. Then, transfected cells were infected as described above for 8 h. The evaluation of silencing was performed by WB at 32 h after transfection.

2.9 | Immunofluorescence analysis

Following transfection and PR8 infection as described above, A549 cells were fixed with methanol, permeabilized with 0.1% Triton X-100, blocked with 3% milk, and stained with anti-HA antibody (Santa Cruz Biotechnology). Alexa-Fluor 546- conjugate anti-mouse was used as a secondary antibody. Nuclei were stained with 4′6-diamidino-2-phenylindole (DAPI).

2.10 | IL-6 ELISA

Human IL-6 was assayed by ELISA (Cusabio), according to the manufacturer's instructions.

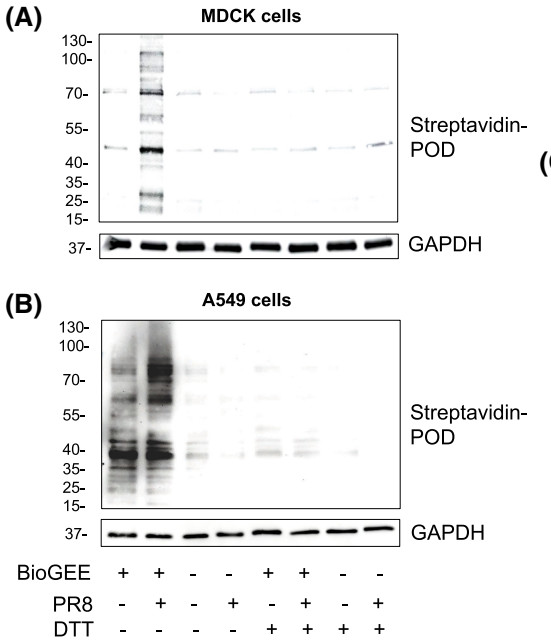
2.11 | Statistical analysis

Differences between the two groups were assessed for statistical significance using a two-tailed Student's t test. A p value <.05 was considered statistically significant.

3 | RESULTS

3.1 | Influenza viruses induce protein glutathionylation

To investigate whether the influenza virus induces protein glutathionylation, we first used MDCK epithelial cells, known to be highly permissive to this virus. Cells were infected with PR8 and treated with BioGEE. After 24 h, cell lysates were analyzed by SDS-PAGE in either non-reducing or reducing conditions, followed by WB with streptavidin-POD to visualize glutathionylated proteins. As shown in Figure 1A, protein glutathionylation was higher in the PR8-infected samples compared to the control. In the lanes either with DTT-reduced samples or with no- BioGEE samples very faint bands were visible, indicating that the signal was due to a binding of BioGEE via a disulfide bond, as in protein glutathionylation, and that there was no non-specific binding of streptavidin to the proteins (Figure 1A). The same experiment was performed in PR8-infected A549 cells with similar results (Figure 1B). To confirm the higher amount of glutathionylated proteins in influenza virus-infected A549 cells, a different technique was used: the mixed disulfides were measured by HPLC and



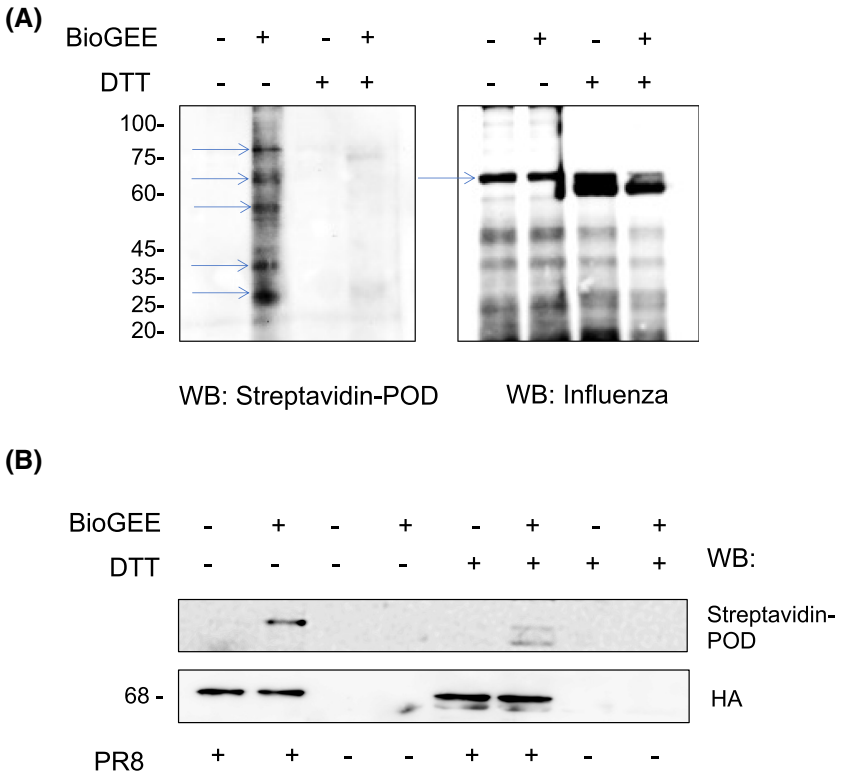
(C) A549 cells

Samples	Mixed Disulfides (nmol/mg protein)
CTR	ND
PR8	0.33 ± 0.07

ND Not detectable

FIGURE 1 Influenza virus induces glutathionylation of proteins in epithelial cells. BioGEE-treated, PR8-infected (or not) MDCK (A) and A549 (B) cell lysates were analyzed by SDS-PAGE in non-reducing (DTT−) and reducing conditions (DTT+), followed by Western blot with streptavidin-POD. Two Western blots representative of six ($n = 3$ biological replicates for each cell line) were shown. GAPDH was shown as the loading control. (C) PR8-infected and not (CTR) A549 cell lysates were analyzed for the measurement of mixed disulfides by HPLC. Data are the mean ± SD of six replicates from two different experiments ($n = 6$).

FIGURE 2 Influenza virus hemagglutinin is glutathionylated in A549 cells. (A) BioGEE-treated, PR8-infected A549 cells were immunoprecipitated with anti-influenza antibody and analyzed by SDS-PAGE in non-reducing (DTT−) and reducing conditions (DTT+), followed by Western blot with streptavidin-POD (on the left); the membrane was stripped and reprobed with anti-influenza antibody (on the right). Representative of three replicates ($n = 3$). (B) BioGEE-treated, PR8-infected A549 cells were analyzed as in (A) but lastly stained with a monoclonal anti-HA antibody.



they were only detectable in cells infected by PR8 virus (Figure 1C).

We then studied RAW264.7 mouse macrophages infected with PR8, NWS, and pH1N1. We detected viral production by the HA assay in the supernatants in a dose-dependent manner 24 h post-infection (p.i.), especially for NWS and pH1N1 strains (Figure S1A). Interestingly, the total amount of free GSH decreased following infection

(Figure S1B) and the signal related to glutathionylated proteins was higher in cells infected with influenza viruses than in control cells (Figure S1C). The HPLC confirmed that viral infection induced mixed disulfide formation (Figure S1D).

3.2 | Influenza virus proteins are glutathionylated

To investigate whether viral proteins undergo glutathionylation, PR8-infected A549 cells were treated with BioGEE and lysed at 24 h p.i., then immunoprecipitation of influenza virus proteins was performed. The immunoprecipitated samples were analyzed by SDS-PAGE in non-reducing or reducing conditions followed by WB with streptavidin-POD. Strong signals from viruses-infected samples were detected, whereas the signals from the same samples reduced with DTT before electrophoresis were considerably decreased (Figure 2A, bands indicated with the arrows in the blot on the left), once again confirming the specific detection of glutathionylated proteins with this technique. Then the membrane was stripped and reprobed with an anti-influenza antibody which confirmed the detection of some proteins that were found glutathionylated in the previous staining (bands over 60 kDa, indicated by the arrow in Figure 2A, blot on the right). The experiment was repeated in the same conditions, but using a monoclonal antibody for viral HA, that has a MW around 68 kDa and we could confirm that HA was glutathionylated in PR8-infected epithelial cells (Figure 2B).

3.3 | Glutaredoxin 1 expression and activity increase in influenza virus infection

The expression of Grx1, the main enzyme catalyzing protein deglutathionylation,[19] was analyzed during viral infection. Grx1 mRNA levels, measured by

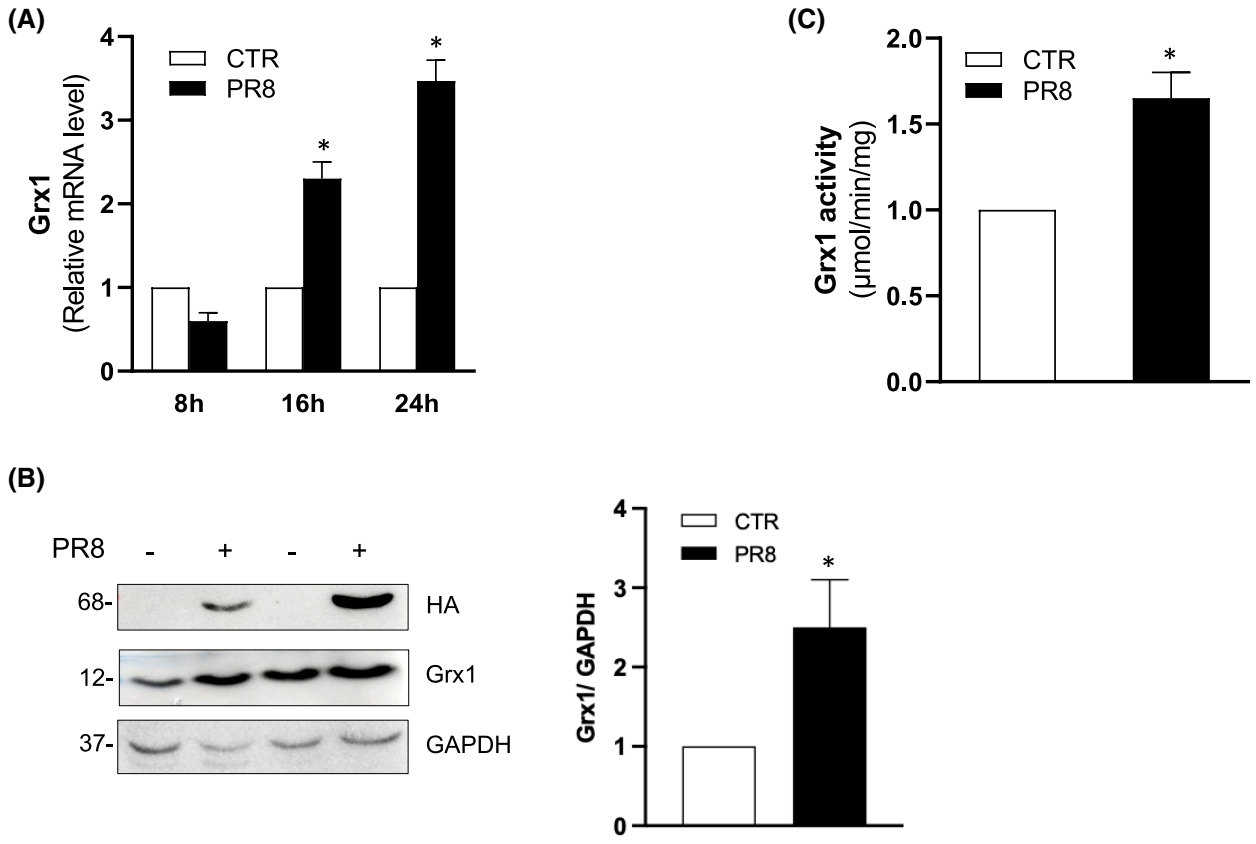


FIGURE 3 Grx1 Expression and activity increase in influenza virus infection. (A) RT-qPCR analysis of Grx1 mRNA level, normalized to actin mRNA level in PR8-infected A549 cells (8, 16, 24 h p.i.). Data are the mean ± SD of three replicates ($n = 3$) (B) Western blot analysis of Grx1 expression in PR8-infected A549 cells 24 h p.i. GAPDH was used as a loading control and HA as a marker of infection. A representative Western blot with two replicates of three was shown and the mean densitometric analysis Grx1/GAPDH was reported in the graph on the right ($n = 3$, $^*p < .05$). (C) Grx1 activity was measured by HED assay at 24 h p.i. Data are the mean ± SD of samples from three experiments, each analyzed in duplicate ($n = 3$, $^*p < .05$).

RT-qPCR, were similar in cell lysates from infected and non-infected cells at 8 h, while increased in infected cells from 16 to 24 h p.i. (Figure 3A). Western blot analysis showed a 2.5-fold increase in the expression of the enzyme in infected cells compared to non-infected cells at 24 h p.i. (Figure 3B). The deglutathionylation activity of Grx1 was also measured by the HED assay,[34] and higher activity in infected cells at 24 h p.i. was confirmed (Figure 3C). These results suggested that the activity of Grx1 and the deglutathionylation process may be required in the late stages of infection to allow the virus to complete its life cycle.

3.4 | Glutaredoxin 1 inhibition increases protein glutathionylation

To investigate whether Grx and hence glutathionylation/deglutathionylation, could have a biological role in viral replication, we blocked Grx1 activity using a known Grx1 inhibitor, 2-AAPA.[33] In preliminary experiments, 2-AAPA toxicity in A549 cells was evaluated by treating the cells with different concentrations of the chemical, from 25 to 200 μM, for 20 min; then the medium was changed and after 24 h the cells were detached and the viability measured by Trypan Blue staining. The percentage of cell death (indicated by the ratio dead/dead+live cells) was less than 10% up to a 2-AAPA concentration of 100 μM, which was therefore chosen for the experiment (Figure S2). As expected, 2-AAPA increased protein glutathionylation in A549 cells (Figure 4A). Then 2-AAPA pre-treated cells were infected with the PR8 virus for 24 h. As shown in Figure 4B, 2-AAPA increased HA glutathionylation (the densitometric analysis revealed a 1.5-fold increase) and the viral titer, evaluated by the HA assay in supernatants 24 h p.i. (Figure 4C), was lower in 2-AAPA-treated cells, showing that Grx1 inhibition increased HA glutathionylation, and this correlated with a decreased viral titer.

3.5 | Glutaredoxin 1 inhibition decreases influenza virus replication

Taking into account that a dynamic process was being evaluated, the second set of experiments was performed

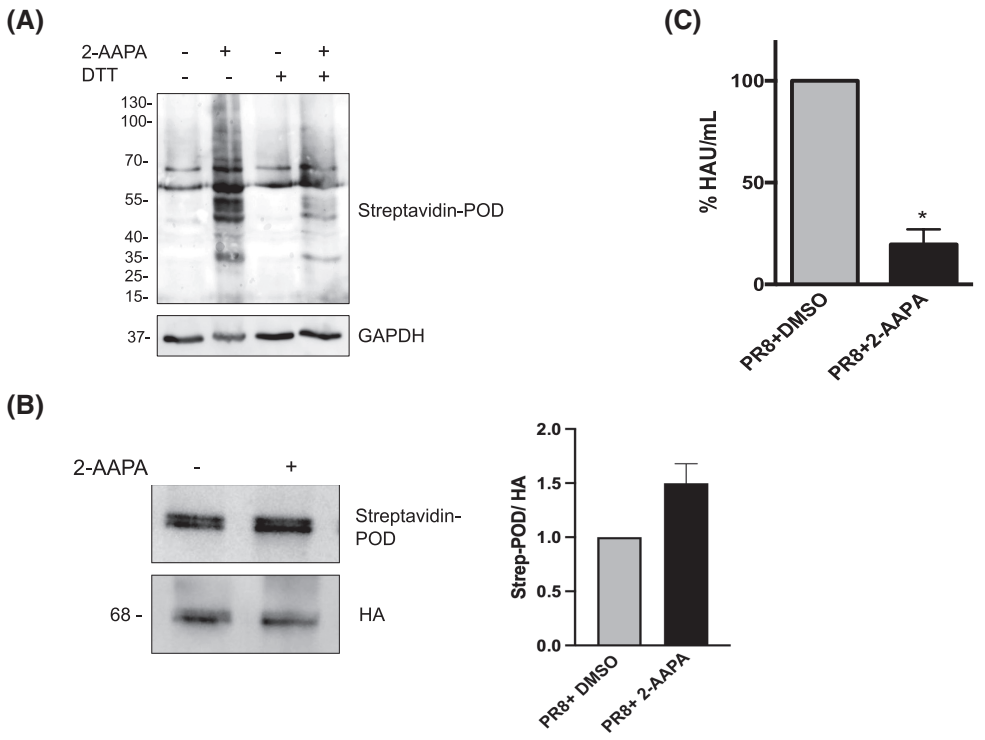


FIGURE 4 Grx1 inhibition by 2-AAPA increases the glutathionylation of proteins. (A) Western blot analysis with streptavidin-POD of BioGEE loaded and 100 μM 2-AAPA treated (or not) A549 cells, in non-reducing (DTT−) and reducing conditions (DTT+). GAPDH was shown as the loading control. (B) 100 μM 2-AAPA pre-treated (or not), BioGEE-treated, PR8-infected A549 cells were immunoprecipitated with anti-influenza antibody and analyzed by SDS-PAGE in non-reducing condition followed by Western blot with streptavidin-POD; the membrane was stripped and reprobed with anti-HA antibody. The mean densitometric analysis Strep-POD/HA was reported in the graph on the right ($n = 2$). (C) HAU from supernatants of 100 μM 2-AAPA-pretreated (or not) and PR8-infected A549 cells 24 h p.i., expressed as percentage mean of three experiments in duplicate ($n = 6$, $^*p < .05$).

during the first single replication cycle of the virus (i.e., at high m.o.i. and for 8 h of infection). After confirming the increase of Grx1 expression in A549 cells infected with a high m.o.i. of PR8 virus, at both mRNA and protein levels (Figure S3), we found that Grx1 inhibition by 2-AAPA led to a similar decrease in HAU at 8 h p.i. (Figure 5A) as during the multicycle infection, at 24 h p.i. Moreover, TCID50 from supernatants of 2-AAPA pre-treated and infected cells was lower than from supernatants of infected samples and it was confirmed by a lower number of viral copies measured by RT-qPCR assay (Figure 5A, graphs in the middle and on the right) indicating that Grx1 inhibition decreased viral replication and infectivity. In treated cells, we observed a decrease in viral protein expression at 8 h p.i., considerably for HA and matrix protein (M1), while the mRNA levels, measured for HA and M2, were similar (Figure 5B,C, respectively). Interestingly, IL-6 levels in the supernatants of the same samples were significantly lower when infected cells were pre-treated with 2-AAPA, suggesting that the Grx1 inhibition may also have an anti-inflammatory effect (Figure S4).

As a second approach, we silenced Grx1 expression using a siRNA. After establishing the Grx1-siRNA concentration, A549 cells were transfected and, after 24 h, infected with the PR8 virus for 8 h. The WB analysis for Grx1 confirmed that the enzyme expression decreased 60% in siGrx1-transfected cells compared to cells transfected with a control non-targeting siRNA; to note that virus infection induced Grx1 increase, as shown in Figures 3 and S3, but the silencing of Grx1 was also efficient in infected cells (Figure 6A). The immunoblotting with anti-influenza and anti-M2 antibodies revealed a decrease in viral protein expression in siGrx1-transfected and infected cells (Figure 6A) similar to what was observed in 2-AAPA-treated infected cells (Figure 5B). The TCID50 and the viral copies from supernatants of the same samples showed a lower viral titer (Figure 6B,C). Immunofluorescence analysis was performed in siGrx1/PR8-infected A549 cells, staining viral HA (red) and cell nuclei (DAPI, blu). As shown in confocal microscopy images (Figure 6D), HA expression was lower in cells where Grx1 was silenced. Accordingly, the HAU in siRNA-treated cells was 12 compared to 32 in the infected samples. Finally, a WB analysis of two markers of endoplasmic reticulum (ER) stress, ATF6 and IRE1 revealed a strong activation of the stress sensors in infected cells,

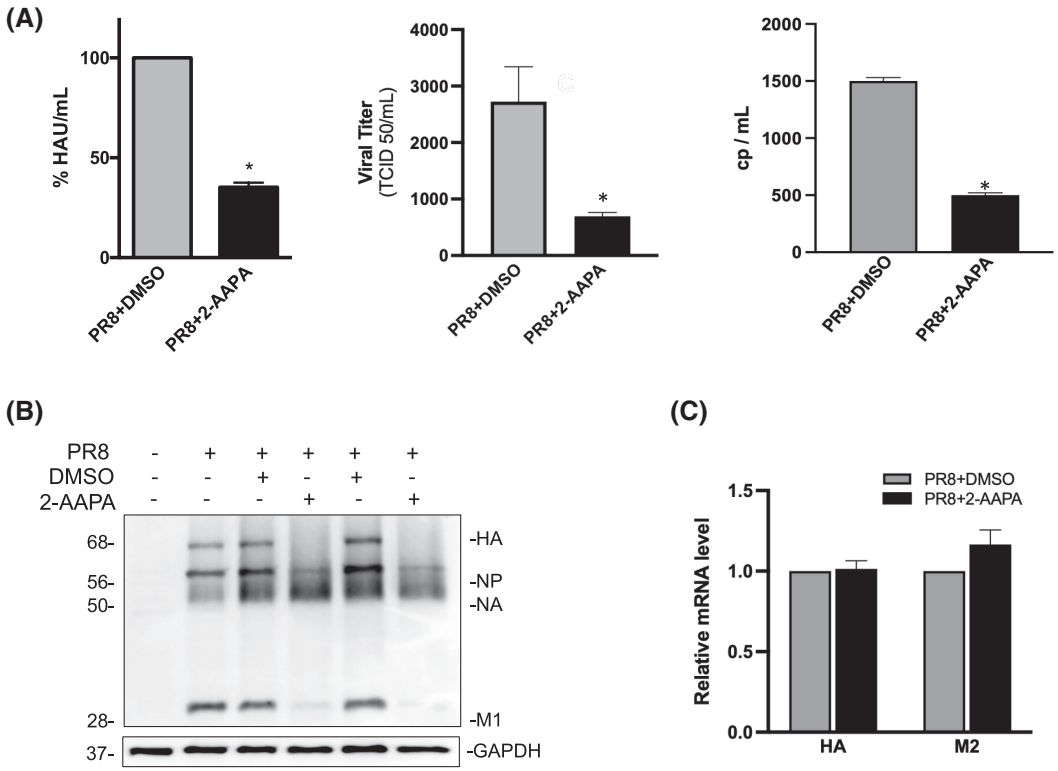


FIGURE 5 2-AAPA inhibits viral replication. (A) Different techniques of influenza viral titration: On the left, HAU from supernatants of 100 μM 2-AAPA-pretreated (or not) and PR8-infected A549 cells 8 h p.i. expressed as percentage mean of six replicates from three independent experiments ($n = 6$, $^*p < .05$); in the middle, TCID50 from supernatants, on the right, viral copies in supernatants quantified by RT-qPCR of the same samples; $^*p < .05$. (B) Western blot analysis of influenza virus proteins in cell lysates of samples as above with anti-influenza antibody. GAPDH was used as a loading control and a representative western blot with two replicates of eight ($n = 8$) was shown. (C) RT-qPCR analysis of HA and M2 mRNA level, normalized to actin mRNA of the same samples.

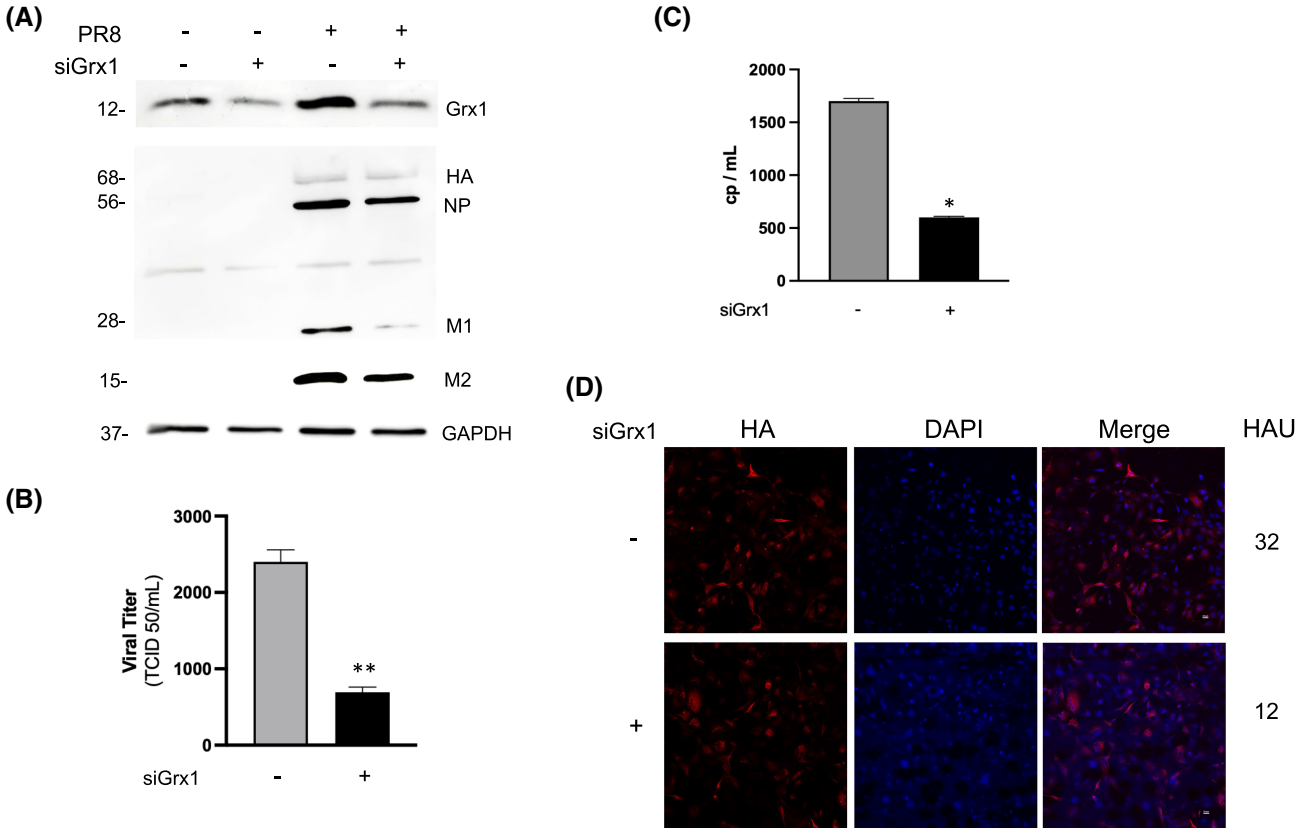


FIGURE 6 Grx1 downmodulation decreases viral replication. (A) Cell lysates of A549 cells transfected with siRNA specific to Grx1 (siGrx1) or with a control non-targeting siRNA and then infected with PR8 virus for 8 h were analyzed by western blot with anti-Grx1, anti-influenza, and anti-M2 antibodies. GAPDH was used as a loading control and a representative western blot of three replicates ($n = 3$) was shown. (B) TCID50 from supernatants of samples as in (A), $^{**}p < .01$ (C) Viral copies in supernatants quantified by RT-qPCR (D) Confocal images of viral HA, nuclei (DAPI) and their merge of the samples as in (A); HAU from their supernatants are indicated on the right of the figure.

as indicated by the cleaved form of ATF6 and the phosphorylation of IRE1; interestingly, activation was lower in siGrx1-transfected and infected cells (Figure S5), as it was lower the accumulation of viral proteins. Therefore, Grx1 downmodulation decreased viral replication, suggesting that deglutathionylation is required for the proper viral maturation and budding from infected cells.

4 | DISCUSSION

In the present study, we showed that (i) influenza viruses induce protein glutathionylation, including that of viral proteins; (ii) Grx1 activity is important for the virus to complete its replication cycle.

Previous studies have shown that different viruses, such as parainfluenza virus, HIV, HSV, and recently, SARS-CoV-2,[20–23] induce glutathionylation of cellular proteins during infection. Using a biotinylated GSH derivative, BioGEE, already used in our previous works,[32] we were able to detect glutathionylated proteins in two PR8-infected epithelial cell lines and influenza virus strains-infected macrophage cells. The latter, essential for protection against influenza, being among the first cells to respond to the virus in the respiratory tract[35,36] have long been considered not to support viral replication; however, recent studies show that the replicative efficiency in macrophage cells depends on the viral strains,[37,38] as we observed in our experiments. Interestingly, the strain with higher replicative efficiency (NWS) induced higher levels of protein glutathionylation. The presence of glutathionylated proteins in the two cell types (epithelial/macrophages), confirmed by HPLC, led us to believe that the glutathionylation is a common mechanism, activated by different viruses in different cells, that is part of the virus-induced redox imbalance, whose relevance is only partially understood.[20–23]

Other studies have demonstrated that viral proteins such as retroviral protease, chikungunya virus nsP2, dengue and zika NS5, HCV polymerase, and SARS-CoV-2 protease can undergo glutathionylation.[24–28] Among influenza virus proteins, we found that HA can be glutathionylated. HA is one of the main viral surface glycoproteins,

rich in cysteines, whose folding starts, with the formation of disulfide bonds, in the ER, before the entire polypeptide has been synthesized.[39] During the biosynthesis of HA, the first cysteine residue to enter the ER lumen could interact with other cysteines, including that of GSH through transient glutathionylation, before its proper cysteine partner is added to the nascent polypeptide.[40] Therefore, glutathionylation could assist the process of folding and maturation of the glycoprotein along the secretory pathway before its insertion into the cell membrane. In fact, mixed disulfides with GSH are believed to be important folding intermediates and Grxs to have a role in facilitating protein folding.[41,42]

These results raise the question of the biological relevance of glutathionylation during viral infection. To address this point, we analyzed Grx1 expression/activity and the effect of its inhibition or silencing on viral replication. A time course analysis of Grx1 during viral infection revealed an increase in the enzyme and its deglutathionylation activity, evaluated by the HED assay, at the late stages of the viral life cycle.[34] This could represent a cell host response to the virus induced-oxidative stress with the formation of mixed disulfides, but it could also have a role in the progression of the virus life cycle. Indeed, it had already been shown that Grx (also known as thioltransferase) can regulate the activity of glutathionylated HIV-1 protease in vitro.[30] Interestingly, glutathionylation could prevent premature activation of the protease; this would be activated and Grx-mediated deglutathionylation could allow its activation later, at the optimal timing for viral maturation and release.[43] Moreover, it was reported that human Grx1 catalyzes the reduction of HIV-1 gp120 disulfides in vitro and that its inhibition reduces viral replication.[44] In our experiments, inhibition of Grx1 by 2-AAPA,[33] or its silencing with a siRNA, increased protein glutathionylation while decreased virus replication, measured as a reduced viral titer in the supernatants, therefore, indicating that Grx1 actually supports the life cycle of the virus.

Interestingly, treatment with 2-AAPA also decreased IL-6 secretion from infected- epithelial cells, suggesting an anti-inflammatory in addition to an antiviral effect. An effect of 2-AAPA on pro-inflammatory cytokine production had been described in *Borrelia burgdorferi* infection, where 2-AAPA reduced the secretion of TNF and IL-1β[45]; however, IL-6 decreased secretion could be a consequence of the reduced viral production, and studies on the role of Grx1 in the inflammatory response to the virus are in progress.

In cells, Grx1 inhibition/downmodulation led to a reduced viral proteins accumulation and a reduced ER stress markers activation as well, while the viral mRNA levels remained unchanged; this observation suggested that the antiviral effect was either co- or post-translational, affecting the proper folding and assembly of viral proteins into new virions, which could thus undergo degradation[46–48] although further studies will be necessary to clarify the pathways involved.

In conclusion, this study shows that glutathionylation is an important regulator of the life cycle of the influenza virus, highlighting the importance of Grx1 in the process; these results might help to define new therapeutic targets to control viral infections.

AUTHOR CONTRIBUTIONS

Paola Checconi designed the study. Paola Checconi, Cristiana Coni, Dolores Limongi, Sara Baldelli, and Marta De Angelis performed the experiments and analyzed the data. Paola Checconi drafted the manuscript. Fabio Ciccarone, Manuela Mengozzi, Pietro Ghezzi, and Maria Rosa Ciriolo interpreted the data and reviewed the manuscript. Lucia Nencioni and Anna Teresa Palamara supervised the research and reviewed the manuscript. All authors read and approved the final manuscript.

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DISCLOSURES

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study were obtained by the analysis described in the methods, further supported by the supplementary material. All data are available on request from the corresponding author.

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