Inhibition of Influenza A Virus Replication by Resveratrol

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We have previously shown that the life cycles of several viruses are influenced by host-cell redox states. Reports of the antioxidant activities of the plant polyphenol resveratrol (RV) prompted us to investigate its effects on influenza virus replication in vitro and in vivo. We found that RV strongly inhibited the replication of influenza virus in MDCK cells but that this activity was not directly related to glutathione-mediated antioxidant activity. Rather, it involved the blockade of the nuclear-cytoplasmic translocation of viral ribonucleoproteins and reduced expression of late viral proteins seemingly related to the inhibition of protein kinase C activity and its dependent pathways. RV also significantly improved survival and decreased pulmonary viral titers in influenza virus–infected mice. No toxic effects were observed in vitro or in vivo. That RV acts by inhibiting a cellular, rather than a viral, function suggests that it could be a particularly valuable anti-influenza drug.

Influenza viruses are enveloped viruses with segmented, single-stranded, negative-sense RNA genomes [1]. Every year, influenza epidemics cause numerous deaths and millions of hospitalizations, but the most frightening effects are seen when new strains of the virus emerge, causing worldwide outbreaks of infection. Recent reports of direct avian-to-human transmission of influenza make the prospect of a new pandemic particularly alarming [2, 3]. The replication of influenza virus has been studied in depth, and several antiviral compounds have been developed, but their long-term efficacy is often limited by toxicity and the almost inevitable selection of drug-resistant viral mutants [4].

Resveratrol (RV; 3,5,4′-trihydroxy-*trans*-stilbene) is a polyphenol that is synthesized by at least 72 plant species, including grapes (50–100 μg/g of RV) and other fruits [5, 6], in response to physiological stimuli and environmental stress [7]. Its health benefits include cardio- and neuroprotective effects and anticarcinogenic activity [8–11]. Some researchers have reported that RV also inhibits the replication of herpes simplex virus and synergistically enhances the effects of known anti-HIV drugs, but the mechanisms underlying these actions remain obscure [12–14]. RV appears to be capable of interfering with several intracellular signaling pathways, including those activated by protein kinase C (PKC) and by mitogen-activated protein kinases (MAPKs) [15–20]. It has documented antioxidant activity [21], and its cardioprotective effects have been related to its inhibition of lipid peroxidation and the oxidation of low-density lipoproteins [5, 22].

There is increasing evidence that the oxidoreductive (redox) balance of cells is involved in viral infections and that certain antioxidant molecules exert potent antiviral activities in vitro and in vivo. We have previously demonstrated that RNA and DNA viruses can deplete host-cell levels of the antioxidant glutathione (GSH) and that the administration of exogenous GSH inhibits viral replication in several experimental systems [23–28]. These observations prompted us to investigate RV's potential for inhibiting the replication of influenza virus and the possible mechanisms underlying these effects.

We found that RV strongly inhibits the replication of influenza A virus in vitro but that this effect did not seem to be directly related to GSH-mediated antioxidant activity. Instead, it appeared to involve the blockade of nuclear-cytoplasmic translocation of viral ribonucleoproteins (vRNPs) and reduced expression of late viral proteins, and these effects were related to the inhibition of PKC activity and its dependent pathways. In in vivo studies, RV also improved survival and decreased pulmonary viral titers in influenza virus–infected mice.

MATERIALS AND METHODS

Cells. MDCK and NCI-H292 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco). Cell viability was estimated by trypan blue exclusion.

Reagents. RV (molecular weight, 228.2 kDa), dissolved in dimethyl sulfoxide (DMSO), was diluted to final concentrations in RPMI 1640 medium (in vitro studies) or PBS (in vivo studies). The highest DMSO concentration in culture medium was 0.02%. RV, buthionine sulfoximine (BSO), and 12-*O*-tetradecanoylphorbol13-acetate (TPA) were purchased from Sigma.

Virus infection. Influenza virus A/Puerto Rico/8/34 H1N1 (PR8) was grown as described elsewhere [24]. Twenty-four hours after plating, cells were challenged for 1 h at 37°C with PR8 at MOI 0.2 (or, when stated, MOI 10), carefully washed, and incubated with medium supplemented with 2% FBS. Mock infection was performed with allantoic fluid of uninfected eggs. Virus production was determined in cell supernatants by measuring hemagglutinin units (HAU) and 50% cytopathic effect (CPE_{50}) at different times after infection, according to standard procedures [29, 30].

Immunoblotting. Cell lysates were separated on SDS-polyacrylamide gels, transferred onto nitrocellulose membranes, and stained with primary antibodies (indicated in figure legends). Secondary antibodies were conjugated to horseradish peroxidase (Sigma). Membranes were developed by use of an enhanced chemiluminescence system (Amersham).

Polymerase chain reaction (PCR). Total RNA was extracted from cells as described elsewhere [31]. Randomly primed cDNA obtained by reverse transcription (RT)–PCR was amplified (GenAmp system 2400; Perkin-Elmer) in a PCR mixture (50 μL) that contained hemagglutinin (HA) or M1 primers, as described elsewhere [24]. The products were electrophoresed and visualized by ethidium bromide staining.

Immunofluorescence. Cells were fixed, permeabilized, and stained with 4′,6′-diamidino-2-phenylindole hydrochloride (Bio-Rad) and then were incubated consecutively with primary and fluorescein isothiocyanate–conjugated secondary antibodies (Sigma).

Glutathione assay. Intracellular GSH and its oxidized form (GSSG) were separated by high-performance liquid chromatography [23]. Aliquots of cell lysates were used for determination of total protein levels, as described elsewhere [32].

Mice and treatments. Four-week-old female BALB/c mice (average weight, 20 g; Charles River) were housed and studied under Institutional Animal Care and Use Committee–approved protocols. Mice were inoculated intranasally with PR8 (2 HAU/mouse) diluted in 50 μL of PBS, as described elsewhere [33], and were randomly divided into experimental groups.

One hour after inoculation, mice received intraperitoneal injections of either RV (1 mg/kg/day) or placebo, and treatments were repeated daily for the next 7 days. Uninfected control groups received identical RV and placebo treatments. Survival was assessed in all groups for 30 days after infection.

Pulmonary viral titers. Two groups of PR8-infected mice treated with RV or placebo were killed 6 days after infection. Each lung was removed, weighed, and homogenized in RPMI 1640 medium for CPE_{50} assay of viral titers.

Statistical analysis. Data are expressed as mean $\pm$ SD, and comparisons were statistically evaluated by analysis of variance (significance level, $P < .05$). Survival curves were compared by the Sign test; $\alpha < .05$ was considered to be significant [33].

RESULTS

Inhibition of PR8 replication in MDCK cells by RV. In all experiments, PR8 replication was evaluated in MDCK cell supernatants 24, 48, and 72 h after infection by HAU and CPE_{50} assays. The results of the 2 methods were consistently concordant, indicating that all observed changes pertained to both viral HA production and infectivity. Unless otherwise stated, results are presented exclusively in terms of HAU at 24 h after infection, which were generally confirmed in 48- and 72-h assays. Different concentrations of RV or DMSO (control infected [CI] cells) were added to MDCK cells 1 h after infection and maintained, unless otherwise stated, for the duration of the experiment (72 h after infection). As shown in figure 1*A,* viral replication was significantly and dose-dependently inhibited by RV concentrations of 10–40 μg/mL. Replication was markedly reduced (by 90% $\pm$ 2.5%, vs. CI cells) by 20 μg/mL of RV and was completely blocked by 40 μg/mL of RV. Comparison of the viral growth kinetics of CI cells and RV-treated cultures revealed that inhibition remained stable through 72 h after infection.

No significant cytotoxic effects were observed in uninfected cells exposed to 10–20 μg/mL of RV. Because 40 μg/mL of RV caused marked morphological alterations and decreased cell viability (data not shown), all subsequent experiments were done with 20 μg/mL of RV.

We first attempted to identify the step(s) of the PR8 life cycle that were affected by RV. As shown in figure 1*B,* no significant antiviral effects were detected when isolated PR8 was pretreated for 1 h with RV or when RV was present in cell cultures only during the 1-h phase of viral adsorption. When cells underwent a 24-h preinfection treatment with RV, with drug washout right before viral challenge, virus production decreased nonsignifi-
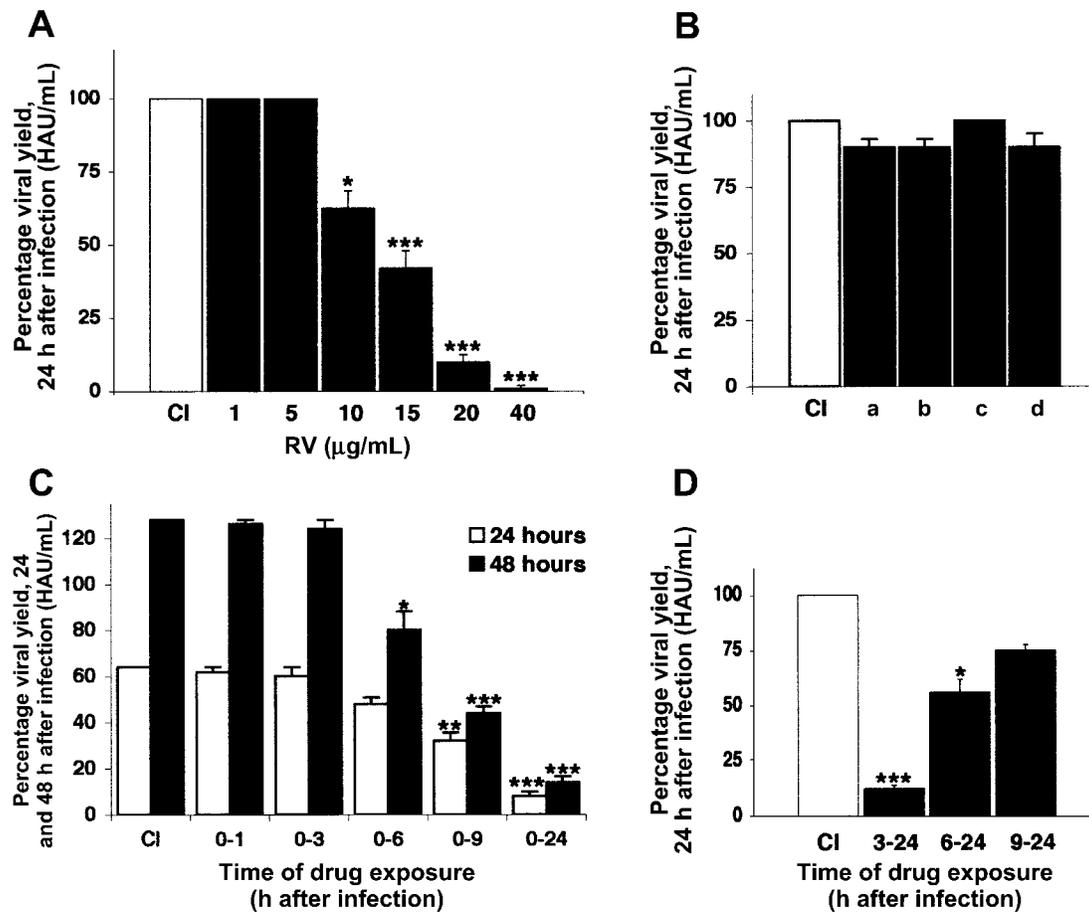
Figure 1. Inhibition of the replication of influenza virus A/Puerto Rico/8/34 H1N1 (PR8) in MDCK cells by resveratrol (RV). *A,* Different concentrations of RV in cell culture medium after PR8 infection (MOI, 0.2). Viral yields 24 h after infection are expressed as percentages of those recorded for control-infected (CI) cells treated with 0.02% dimethyl sulfoxide (DMSO; the concentration present in culture medium containing the highest dose of RV). Values shown are means $\pm$ SD of 4 experiments, each run in duplicate. $n = 4$; $*P = .002$; $***P < .0001$, vs. CI cells. *B,* Viral yields 24 h after infection for CI cells and cells infected/treated as follows: *a* and *b,* Before infection, PR8 was incubated at 37°C for 1 h in medium that contained 0.02% DMSO *(a)* or 40 μg/mL RV *(b)*. *c,* Cells infected with PR8 in the presence of RV (20 μg/mL). The drug was added during viral adsorption and removed after infection. *d,* Cells incubated with RV (20 μg/mL) for 24 h. RV was removed, and cells were infected with PR8. Each value represents the mean $\pm$ SD of quadruplicate samples. *C,* RV (20 μg/mL) was added to cell cultures immediately after PR8 challenge (hour 0) and removed at different time points (1, 3, 6, 9, or 24 h) after infection. The viral yields reported for 24 and 48 h after infection are means $\pm$ SD of 2 experiments, each of which was run in duplicate. $n = 4$; $*P = .002$; $**P = .001$; $***P < .0001$, vs. CI cells. *D,* Addition of RV 3, 6, or 9 h after infection. This was maintained in the culture medium for 24 h after infection. The viral yields 24 h after infection are expressed as percentages of CI values. Each value is the mean $\pm$ SD of 2 experiments, each of which was run in duplicate. HAU, hemagglutinin units. $n = 4$; $*P = .002$; $***P < .0001$, vs. CI cells.

cantly. When cells received combined RV treatment 24 h before and after infection, viral yields 24 h after infection (data not shown) were not significantly lower than those observed with postinfection treatment alone (figure 1*A*), which confirmed that the pretreatment of uninfected cells did not significantly modify their susceptibility to PR8 infection. Overall, these data suggest that RV's antiviral effects involve neither virus inactivation nor the inhibition of viral adsorption.

Next, RV was added to cell cultures immediately after virus challenge and then removed at different time points (figure 1*C*). Postinfection RV treatment for 1 or 3 h produced no significant antiviral effect, which confirmed that RV does not act by pre-

venting virus entry into the cells. In cells exposed to RV for the first 6 h after infection, slight decreases (25% $\pm$ 3% and 37.5% $\pm$ 8%) in viral replication were noted 24 and 48 h after infection. More substantial inhibition was observed when exposure was extended to the first 9 h (result after 24 h, 50% $\pm$ 3.5%; result after 48 h, 65.6% $\pm$ 3%) or 24 h after infection (result after 24 h, 87.5% $\pm$ 2%; result after 48 h, 89% $\pm$ 2.5%).

In other experiments, RV treatment was started at different times after PR8 infection, and the compound remained in the medium through 24 h after infection (figure 1*D*). Maximum inhibition (87.5% $\pm$ 1.5%) of viral replication was achieved when treatment began 3 h after virus challenge. Effects were much

more limited but were still significant when treatment was delayed until 6 h after infection, but no significant inhibition was noted when the drug was added 9 h after infection. These data indicate that RV's antiviral activity is largely related to its inhibition of virus life-cycle steps that occur 3–9 h after infection (and that are possibly related to posttranscriptional events).

Expression of late viral protein and vRNP traffic after RV treatment. The influenza A viral proteins that are synthesized immediately after infection include 3 polymerases (PB1, PB2, and PA) and nucleoprotein (NP); the 2 major external glycoproteins, HA and neuraminidase, and matrix protein 1 (M1) are late gene products. HA can be found in host cells in an uncleaved precursor form (HA0) or in a cleaved form that consists of 2 disulfide-linked chains (HA1 and HA2) [1].

To determine whether RV's inhibition of viral replication was related to the modulation of viral protein synthesis, we treated monolayers with RV at different concentrations immediately after PR8 infection, separated cell lysates 24 h after infection by SDS-PAGE, and immunostained them with anti-influenza antibodies. A single set of mock-infected cells served as negative controls.

As shown in figure 2A, densitometric analysis revealed decreased expression of HA0, HA1, and HA2 (by 85%–90%, vs. that in untreated controls) and of M1 (by 80%–90% or more, vs. that in untreated controls) by cells treated with 20 μg/mL of RV. These decreases were consistent with a >90% reduction in viral yields in RV-treated cells. The expression of early viral protein was not affected by RV, although mild depression of NP ($\leq$30%) and polymerase ($\leq$40%) was observed with 20 μg/mL of RV. The latter effect was highly variable and may simply have reflected reductions in the number of infected cells caused by RV-induced decreases in viral particle release.

To determine whether RV's inhibition of the expression of late viral protein was related to a block in the transcriptional phase of the virus life cycle, mRNAs for HA and M1 were measured in untreated and RV-treated cells by RT-PCR. Both levels of mRNA were efficiently transcribed in RV-treated cells (figure 2B), although the expression of these proteins and virus production were clearly diminished by treatment, which suggests that RV's effect on viral protein expression occurs at a posttranscriptional level.

During influenza virus replication, viral RNAs are packaged into helical vRNP complexes with polymerase and NP in the host-cell nucleus and are subsequently exported into the cytosol to be assembled with the other structural proteins [34]. The inhibition of M1 and HA expression induced by the protein-kinase inhibitor H7 is reportedly related to its blockage of nuclear-cytoplasmic vRNP translocation [35]. To determine whether the same mechanism is involved in RV's inhibition of the expression of late viral protein, we used immunofluorescence to localize NP in RV-treated cells at different times after infection at a high MOI, to allow single-cycle replication (figure 2C). In untreated cells, NP was located almost exclusively in the nuclei at 4 h after infection; however, by 12 h after infection, it was predominantly cytoplasmic. In RV-treated cells, NP was still largely confined to the nuclei at 12 h after infection. Similar results were observed in cells infected for 24 h with a low MOI, to allow multicycle replication.

Collectively, these results indicate that RV interferes with late viral protein synthesis and nuclear export of vRNPs. We then investigated the molecular mechanisms responsible for such effects.

RV and intracellular redox state. To determine whether RV's anti–influenza virus activity involved modulation of the intracellular redox state, we treated mock-infected and PR8-infected cells with 20 μg/mL of RV for 24 h and assayed the cells for GSH and GSSG levels (figure 3A). PR8 infection alone significantly diminished intracellular GSH levels, but a smaller (albeit significant) decrease also occurred in mock-infected cells treated with RV. GSSG levels were unchanged, which indicates that the decreases in GSH levels were not related to its oxidation. RV treatment of infected cells caused only a slight increase in GSH levels.

Our previous research demonstrated that increased GSH levels are responsible for the inhibition of late viral protein synthesis but not of vRNP export [24]. Therefore, we reevaluated both events in RV-treated infected cells after the BSO-induced inhibition of GSH neosynthesis. As expected, the inhibition of GSH synthesis in untreated PR8-infected cells enhanced the expression of HA and M1. In RV-treated infected cells, BSO abolished the slight increase in GSH levels shown in figure 3A but did not significantly modify the drug's inhibition of viral production, protein expression, and vRNP trafficking (figures 3B and 2C), which suggests that RV's antiviral activity is not directly related to GSH-mediated antioxidant activity.

RV interference with the PKC pathway. Many of RV's biological effects are related to its modulation of the activities of protein kinases, such as PKC, that are involved in intracellular signaling [17–20]. Furthermore, several kinase cascades are activated during influenza virus infection [36]. Because H7, a broad-spectrum kinase inhibitor that also affects PKC activity [37], reportedly inhibits the expression of late viral protein and translocation of nuclear-cytoplasmic vRNP in PR8-infected CHO cells [35], we attempted to determine whether H7 and RV targeted the same steps in PR8 replication in our model. At doses of 30 and 50 μmol/L (which produced an inhibition of PR8 replication similar to that produced by 20 μg/mL of RV [93.7% and 100%, respectively]), H7 strongly inhibited the expression of all viral proteins except polymerase and NP (figure 4A) and markedly reduced vRNP nuclear-cytoplasmic translocation, which suggests that RV and H7 target similar events in the influenza viral live cycle.

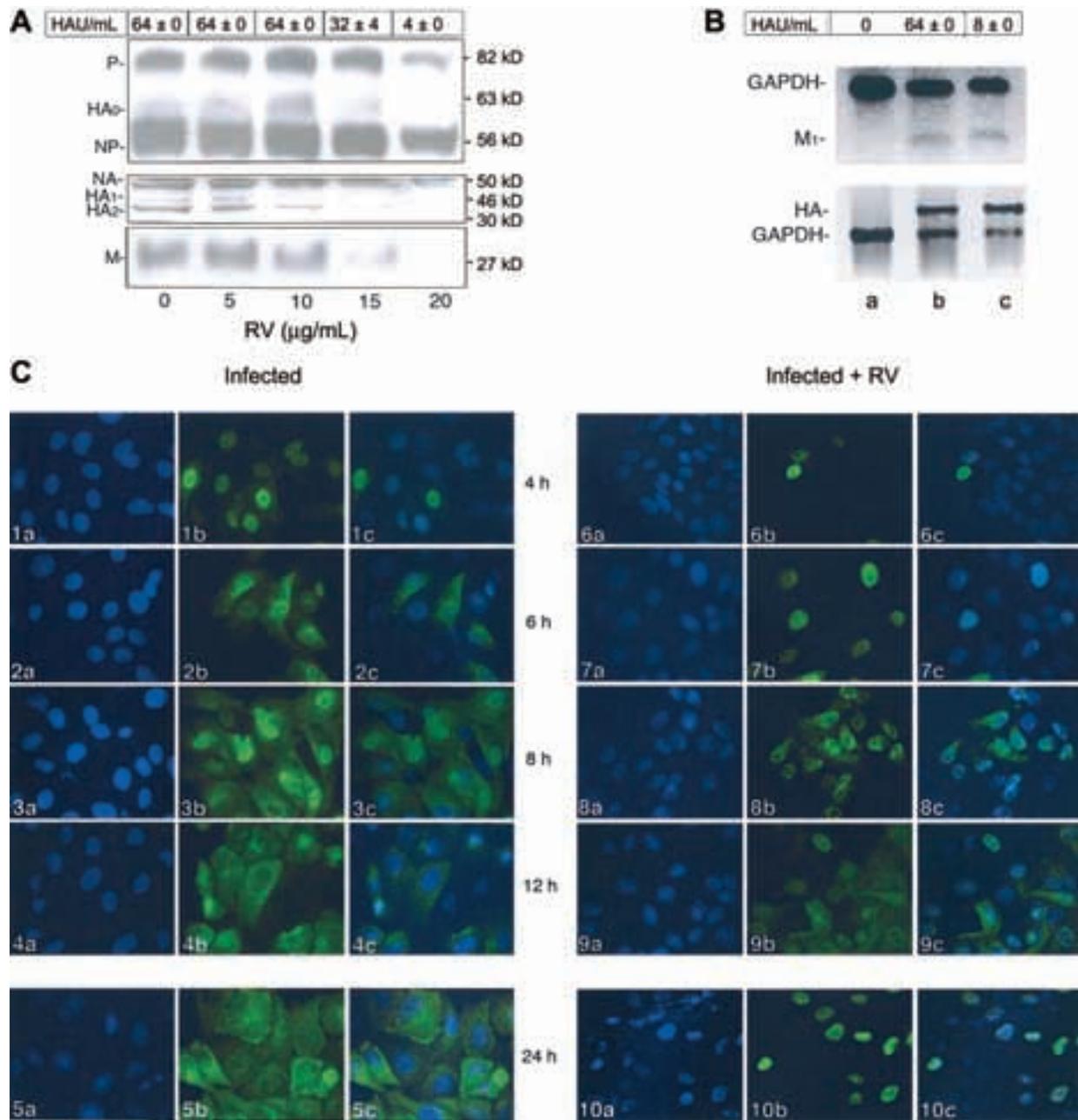We then performed Western-blot analyses to obtain more

Figure 2. Expression of late viral proteins and ribonucleoprotein trafficking during treatment with resveratrol (RV). *A,* Expression of viral proteins in influenza virus A/Puerto Rico/8/34 H1N1 (PR8)–infected cells untreated (RV 0) or treated with RV at different concentrations. After 24 h, cells were lysed; samples were normalized by the Bradford method (Bio-Rad), separated by 12% SDS-PAGE, transferred to nitrocellulose membranes, and immunostained with goat polyclonal anti–influenza A virus antibody (Chemicon). PR8 virus proteins and molecular-weight values are indicated to the left and to the right, respectively, of the figure. *B,* Expression of mRNA for PR8 hemagglutinin (HA) and M1 proteins in mock-infected or infected cells treated with RV (20 μg/mL) or not treated. mRNA extracted 18 h after infection was amplified by polymerase chain reaction (PCR) with a limited no. ($n = 25$) of amplification cycles, analyzed on 1% agarose gel, and visualized and photographed on exposure to UV light. mRNA encoding GAPDH was amplified by PCR under the same conditions. *a,* Mock-infected cells; *b,* Control-infected cells; *c,* RV-treated infected cells. *C,* Intracellular localization of nucleoprotein (NP) in RV-treated and untreated cells at different times after infection. Cells were infected with MOI 10 or 0.2 to allow single-cycle (evaluated 4–12 h after infection) or multicycle (24 h after infection) replication, respectively. Cells were fixed with methanol/acetone (1:2), permeabilized with 0.1% Triton X, stained with monoclonal anti-NP antibody (Oxford Biotechnology) (panels 1–10b), and analyzed by fluorescence microscopy. Nuclei were stained with 4′,6′-diamidino-2-phenylindole hydrochrloride (DAPI; panels 1–10a). Merged images of the fluorescein isothiocyanate and DAPI signals from panels 1–10b and 1–10a are presented in panels 1–10c. The values reported in the upper panels of *A* and *B* represent the viral yields of the supernatants (in HA units [HAU] per milliliter). All results shown are for 1 representative experiment of 3 performed.
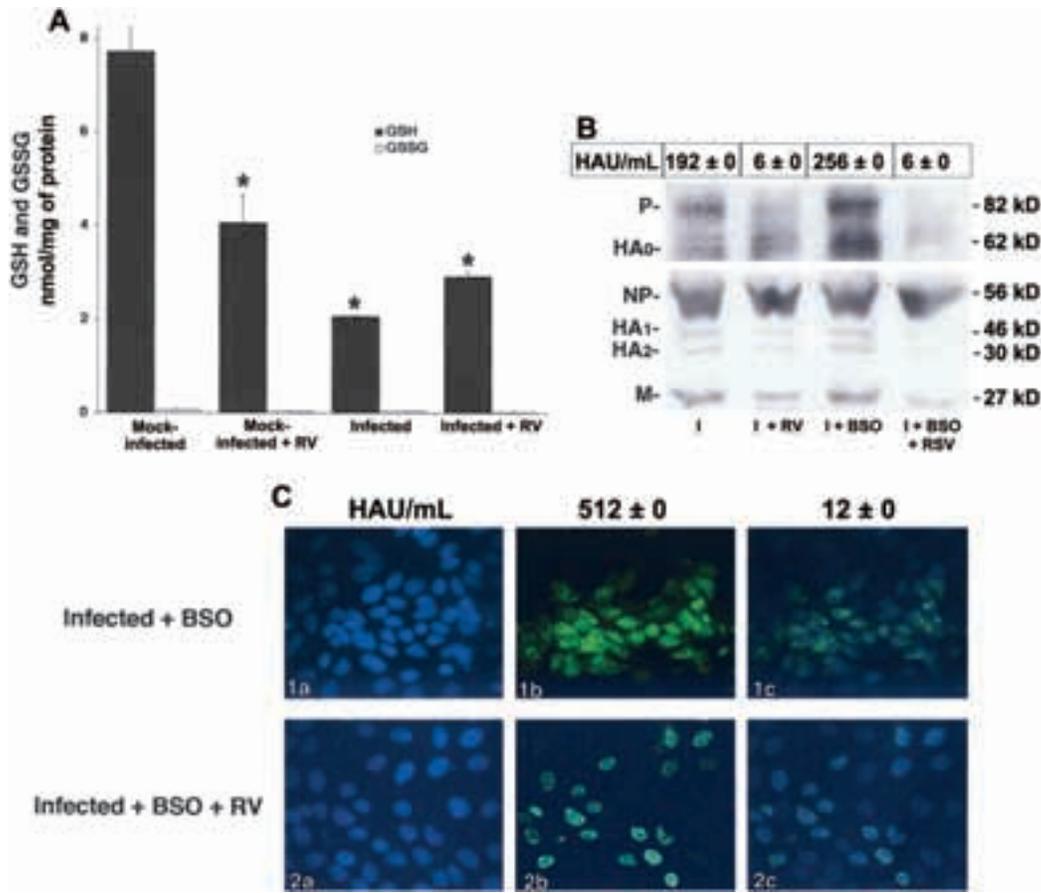
Figure 3. Effect of resveratrol (RV) on the intracellular redox state. *A,* Intracellular glutathione (GSH) and GSSG (oxidized GSH) levels in MDCK cells. Infected or mock-infected cells were treated with RV (20 μg/mL), and intracellular GSH and GSSG levels were assayed by high-performance liquid chromatography (HPLC) 24 h later. Results are expressed as nanomoles per milligram protein of cell extract. Each value represents the mean ± SD of 2 different experiments, each of which was run in duplicate. $n = 4$; *$P < .05$, vs. mock-infected cells. *B* and *C,* Effect of buthionine sulfoximine (BSO) on influenza virus A/Puerto Rico/8/34 H1N1 (PR8) protein expression and intracellular localization of nucleoprotein (NP) in RV-treated and -untreated cells. Cells were exposed to BSO (1 mmol/L) beginning 18 h before PR8 infection (MOI 0.2) through 24 h after infection, which reduced intracellular GSH to levels that were undetectable with HPLC. *B,* Lysis of cells and separation of samples by a 8% *(top)* and 12% *(bottom)* SDS-PAGE. The gels were blotted onto a nitrocellulose membrane and immunostained with anti–influenza A virus antibodies. PR8 virus proteins and molecular-weight values are indicated to the left and to the right of the figure, respectively. HA, hemagglutinin; I, infected cells; M, matrix protein 1; P, polymerase. *C,* Cells fixed with methanol/acetone (1:2), permeabilized with 0.1% Triton-X 100, stained with monoclonal anti-NP antibodies (panels 1b and 2b), and analyzed by fluorescence microscopy. Nuclei were stained with 4′,6′-diamidino-2-phenylindole hydrochroride (DAPI; panels 1a and 2a). Merged images of the fluorescein isothiocyanate and DAPI signals from panels 1a,b and 2a,b are presented in the panels 1c and 2c, respectively. Results shown are for 1 representative experiment of 3 performed. The values reported in the upper panels of *B* and *C* represent the viral yields of the supernatants (in HA units [HAU] per milliliter).

information on the possible involvement of the PKC pathway in RV's antiviral activity (figure 4*C*) in the PR8-infected human cell line NCI-H292, a well-characterized model of influenza virus infection [38]. Because the PKC family includes several serine/threonine kinases that act by catalyzing the phosphorylation of specific substrates [39], we first analyzed the phosphorylation of the PKC downstream effector, PKD [40]. As shown in Figure 4*C*, PKD phosphorylation was observed in infected cells 8 h after infection, and this event was markedly reduced by RV treatment. RV also inhibited TPA-induced PKC

activation, which is consistent with results in other experimental models [18]. Because PKC is reportedly involved in the activation of MAPKs [11], we also analyzed the phosphorylation patterns of p38MAPK, JNK, and ERK1/2. As shown in figure 4*C*, in PR8-infected cells, all 3 MAPKs were activated 8 h after infection. The addition of 20 μg/mL of RV diminished p38MAPK and JNK phosphorylation but had no effect on that of ERK1/2. Similar results were obtained in RV-treated cells stimulated with TPA. These findings probably reflect the differing MAPK-activating effects of the various PKC isoenzymes
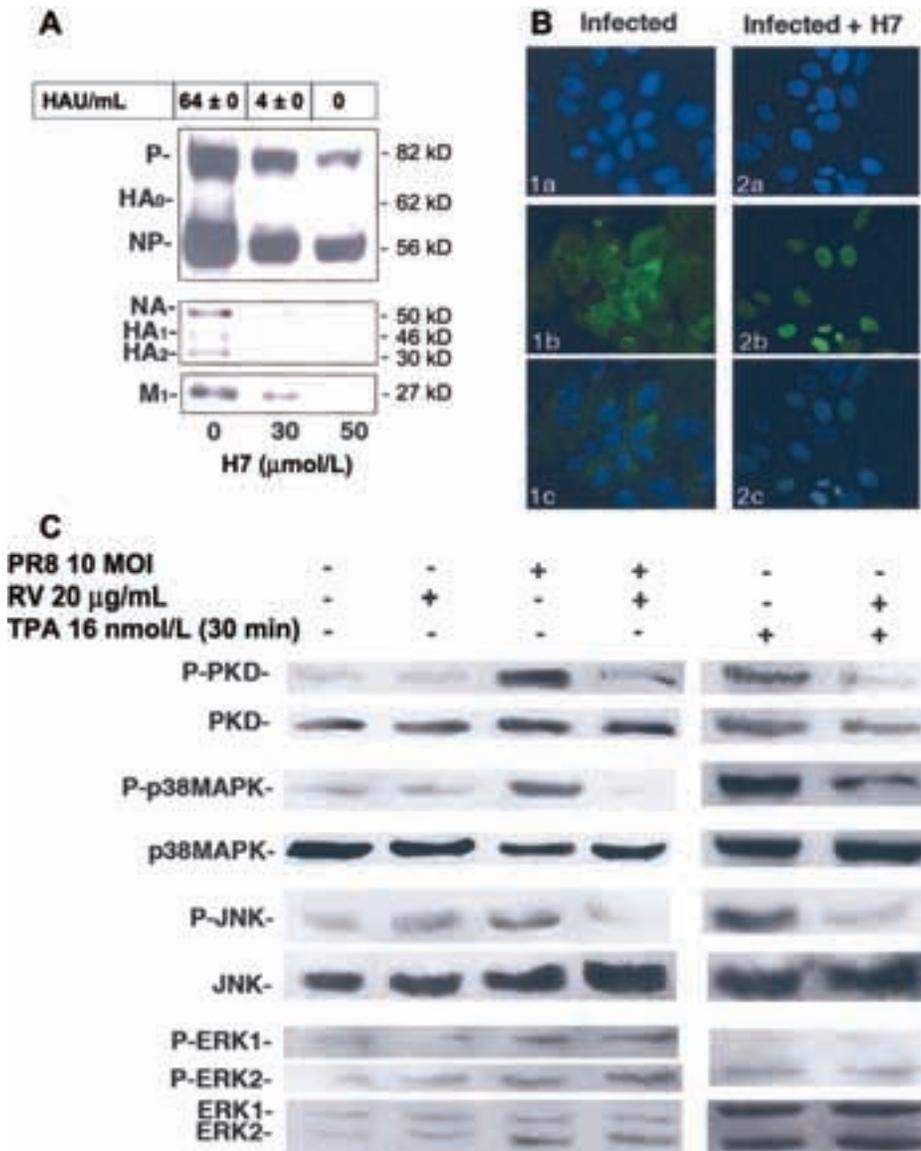
Figure 4. Interference of resveratrol (RV) in the protein kinase C (PKC) pathway. *A,* Effect of H7 on synthesis of viral proteins in MDCK cells. Influenza virus A/Puerto Rico/8/34 H1N1 (PR8)–infected cells treated for 24 h after infection with 30 or 50 μmol/L H7 were lysed and separated by 10% gel SDS-PAGE, transferred to nitrocellulose, and immunostained with anti–influenza A virus antibodies. PR8 protein expression and the corresponding viral yields 24 h after infection *(top)* are shown. Molecular weights are indicated on the right. HAU, hemagglutinin units; NA, neuraminidase; P, polymerase. *B,* Effect of H7 on nucleoprotein (NP) localization in MDCK cells. PR8-infected cells treated for 24-h after infection with H7 (30 μmol/L) were fixed and permeabilized (as described in figure 2), stained with 4′,6′-diamidino-2-phenylindole hydrochrloride (DAPI; panels 1a and 2a), and analyzed by fluorescence microscopy with monoclonal anti-NP antibodies (panels 1b and 2b). Merged images of the fluorescein isothiocyanate and DAPI signals from panels 1a,b and 2a,b are presented in the panels 1c and 2c, respectively. *C,* Effect of RV on PKC and its dependent pathways in NCI-H292 cells. Cells were mock- or PR8-infected (MOI, 10), treated with RV (20 μg/mL) for 8 h after infection, and lysed. Proteins were separated by 10% SDS-PAGE and gels were blotted onto nitrocellulose membranes and immunostained with rabbit anti–phospho-PKD, anti–phospho-ERK1/2, or anti–phospho–p38 mitogen-activated protein kinase (MAPK), followed by anti–PKD, anti-ERK1/2, anti-p38MAPK, or mouse anti–phospho-JNK antibodies followed by anti-JNK antibodies. In separate experiments, uninfected NCI-H292 cells were treated for 1 h with 20 μg/mL RV and then stimulated for 30 min with 16 nmol/L 12-*O*-tetradecanoylphorbol13-acetate (TPA). Lysates were analyzed by Western blotting with the same primary and secondary antibodies listed above. Results shown are for 1 representative experiment of 3 performed.

[41]. Nonetheless, these experiments indicate that RV's antiviral activity may be mediated by the inhibition of PKC and some of its dependent pathways.

RV effectiveness in influenza virus–infected mice. RV's in vivo antiviral activity was evaluated in a well-established murine model of influenza infection [33]. In preliminary experiments, the viral inoculum that we used caused 80% mortality within 10 days after infection, and mice that survived to day 30 were considered to have been cured.

For survival experiments, RV and placebo treatments were administered to 2 groups of infected mice and to 2 groups of uninfected control mice. There were no deaths and no signs of toxicity or weight loss in either of the control groups, but 80% of the infected mice treated with placebo were dead by day 10 after infection (figure 5*A*). RV significantly increased survival (average, 40% vs. placebo-treated control mice). None of the mice that survived to day 10 after infection showed any signs of disease for the next 3 months and were considered to have been cured.

To determine whether the increased survival was associated with decreased pulmonary viral titers, we infected other groups of mice with PR8 and treated them daily with RV or placebo. Six days after infection, mice were killed, and pulmonary viral titers were determined by CPE_{50} assay. As shown in figure 5*B*, the mean titer for the RV-treated group was 98% lower than that for the placebo-treated control mice.

DISCUSSION

We have shown that RV, a natural polyphenol whose concentration in red wine is 1.5–3.0 mg/L [6], can inhibit the in vitro and in vivo replication of influenza A virus without producing any significant toxicity. The drug's effects involved blockade of the nuclear-cytoplasmic translocation of vRNP complexes, decreased expression of late viral proteins, and an inhibition of cellular PKC activity and its dependent pathways.

Depletion of host-cell GSH is a direct consequence of several viral infections, and various antioxidant substances display strong antiviral activities [23–28, 42–45]. RV has been characterized as a potent free-radical scavenger [46], and it has reportedly increased GSH levels in different experimental models [47, 48]. Therefore, our in vitro findings of decreased GSH levels in uninfected cells treated with RV and its mild effect in restoring the GSH depletion provoked by viral infection were somewhat unexpected. However, natural phenols can produce in vivo antioxidant or pro-oxidant effects, depending on their own oxidative status [49], which, in turn, reflects the specific redox potential in the microenvironment [50]. Thus, although RV can quench reactive free radicals by donating hydrogen atoms [51], this process also generates phenoxyl radicals that can oxidize GSH to GS• [52]. Moreover, the oxidation of the RV-phenoxyl radical produces an RV-quinone form, which can
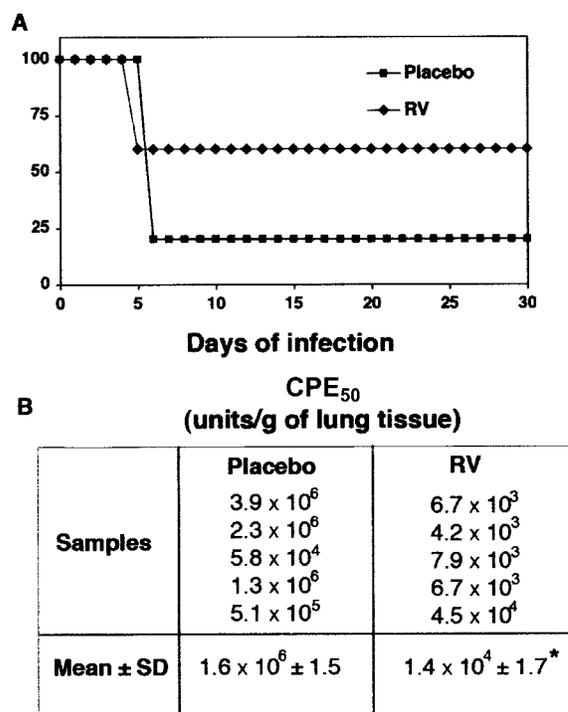
Figure 5. Effect of resveratrol (RV) on influenza virus–infected mice. *A,* Survival. BALB/c mice were infected intranasally with influenza virus A/Puerto Rico/8/34 H1N1 (PR8), and RV or placebo (DMSO diluted in PBS at the same concentrations present in RV injections) were administered intraperitoneally (0.1-mL injection volume) 1 h after virus inoculation (*n* = 10/group) and daily for the next 7 days. Results are expressed as percentage of survival, evaluated daily for 30 days. Survival of RV-treated mice was significantly increased, compared with that of mice treated with placebo ($\alpha < 0.05$). *B,* Pulmonary viral titers. Lungs obtained from RV-treated (20 μg/mouse/day) and placebo-treated mice (*n* = 5/group) were individually homogenized, and virus titers were determined by 50% cytopathic effect (CPE_{50}) assay. Data are expressed as CPE_{50} units per gram of lung tissue. *$P < .05$, vs. placebo. Both experiments (survival and pulmonary viral titers) were repeated 3 times, with identical results.

alkylate GSH and further diminish intracellular concentrations of free GSH. Our findings suggest that RV's inhibition of PR8 replication involves mechanisms other than a GSH-mediated modulation of the cell redox state, although we cannot exclude the possibility that the RV quinone is involved in the drug's inhibition of virus growth [53].

RV had little effect on early viral protein expression, but it dose-dependently inhibited the expression of M1 and HA. That mRNA for these late viral proteins was efficiently transcribed in the presence of RV suggests that the drug acts on posttranscriptional phases of the viral life cycle. This hypothesis was confirmed by immunofluorescence data that showed an RV-induced blockade of the nucleocytoplasmic translocation of vRNPs. Inhibition of influenza virus replication, with decreased production of HA and M1 and nuclear retention of vRNPs, is also produced by the broad-spectrum kinase inhibitor H7 [35],

and this similarity suggests that kinase inhibition might be involved in RV's antiviral effects.

Influenza A virus infection causes the activation of various MAPK pathways [36], including the p38MAPK and JNK pathways (which are thought to play roles in the inflammatory and apoptotic responses [54–56]) and the Raf/MEK/ERK cascade. Blockade of the latter pathway with the ERK inhibitor U0126 results in nuclear retention of vRNPs and diminished virus production but has no effect on the expression of late viral protein [57]. Phosphorylation events also seem to play crucial roles in other steps of the influenza virus life cycle, such as cell penetration and budding [58, 59].

The influenza A virus has 6 phosphorylated proteins, including NP [60]. That both H7 and U0126 block the export of vRNPs to the cytosol strongly suggests that a phosphorylation event is required for efficient nuclear export of NP, but the specific kinase responsible for this event has not been identified [61]. Pleschka et al. [57] reported that NP phosphorylation is not directly affected by the ERK inhibitor U0126 and suggested that vRNP export might even depend on the phosphorylation of a cellular factor.

RV reportedly interferes with signaling cascades by modulating the activities of kinases and other enzymes—for example, the inhibition of PKC activity [17, 18]. It also exerts modulatory effects on MAPK pathways as a consequence of the inhibition of PKC activity [11, 15]. In the present study, RV efficiently inhibited the PR8- and TPA-induced phosphorylation of PKD, a downstream effector of PKC, as well as that of p38MAPK and JNK. That ERK activation was not affected by RV is consistent with previous observations [62] and suggests that (1) different PKC isoenzymes can be involved in the activation of different MAPK pathways and (2) the functional outcome is both isoenzyme and cell-type specific [41]. Our data strongly suggest that RV's antiviral effects are related to the inhibition of PKC activity and its dependent pathways. Studies are already under way to identify the cellular and/or viral substrates of RV-inhibited kinases and their specific roles in the PR8 life cycle.

RV's in vitro antiviral effects were mirrored in a murine model of influenza. Treatment of PR8-infected mice markedly improved their survival, decreased pulmonary virus titers, and caused no significant toxicity. The latter finding is consistent with the results of previous in vivo studies, including some in which RV was administered at doses higher than the ones that we used [48]. Different mechanisms might underlie the in vivo efficacy of RV documented in our study. RV inhibits several cell-signaling pathways [15, 20] that are involved in the inflammatory airway damage that is characteristic of influenza disease [36]. This finding raises the possibility that the survival benefits of RV observed in our study involved a dual mechanism: inhibition of both viral replication and NF-κB–induced inflammation. Studies under way in our laboratory of inflammatory-cytokine levels in PR8-infected mice treated with RV should shed more light on this hypothesis.

All currently approved anti-influenza drugs target essential viral functions and/or structures, and the major drawback of this approach is that the virus will eventually adapt to the selective pressure exerted by the drug [36]. Inactivation of host-cell functions that are essential for virus replication, which seems to be the mechanism of RV's anti-influenza activity, offers 2 important advantages: not only it is more difficult for the virus to adapt to, but it can also be expected to affect viral replication independently of the invader's type, strain, and antigenic properties. For these reasons, RV merits further investigation as a potential weapon for combating the growing threat of influenza.

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