Decreased Intracellular Superoxide Levels Activate Sindbis Virus-induced Apoptosis*

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Infection of many cultured cell types with Sindbis virus (SV), an alphavirus, triggers apoptosis through a commonly utilized caspase activation pathway. However, the upstream signals by which SV activates downstream apoptotic effectors, including caspases, remain unclear. Here we report that in AT-3 prostate carcinoma cells, SV infection decreases superoxide (O$_2^-$) levels within minutes of infection as monitored by an acenitase activity assay. This SV-induced decrease in O$_2^-$ levels appears to activate or modulate cell death, as a recombinant SV expressing the O$_2^-$ scavenging enzyme, copper/zinc superoxide dismutase (SOD), potentiates SV-induced apoptosis. A recombinant SV expressing a mutant form of SOD, which has reduced SOD activity, has no effect. The potentiation of SV-induced apoptosis by wild type SOD is because of its ability to scavenge intracellular O$_2^-$ rather than its ability to promote the generation of hydrogen peroxide. Pyruvate, a peroxide scavenger, does not affect the ability of wild type SOD to potentiate cell death; and increasing the intracellular catalase activity via a recombinant SV vector has no effect on SV-induced apoptosis. Moreover, increasing intracellular O$_2^-$ by treatment of 3T3 cells with paraquat protects them from SV-induced death. Altogether, our results suggest that SV may activate apoptosis by reducing intracellular superoxide levels and define a novel redox signaling pathway by which viruses can trigger cell death.

Apoptosis is a genetically controlled process, with distinct morphological and biochemical features, by which cells commit suicide during development and in disease states (1, 2). Sindbis virus (SV), 1 an alphavirus, belongs to the growing list of viruses (3–10) that can trigger apoptosis in infected host cells in vitro or in vivo. The elimination of virus-infected cells via apoptosis is believed to be a mechanism used by the host to limit the spread of progeny virus to neighboring cells and thus to decrease the host viral burden (11). However, if the primary targets in vivo are nonreplenishing cells like neurons, virus-induced apoptosis can also have devastating consequences for the host. For example, age-dependent encephalitis and mortality induced by SV is correlated with the ability of SV to induce apoptosis in neurons in the central nervous system of mice (12, 13).

Mechanistic studies of SV-induced apoptosis indicate that SV engages an apoptotic pathway used by many death stimuli (14). CrmA, a caspase-inhibitory protein encoded by cowpox virus (15), inhibits SV-induced apoptosis in cultured cells and reduces SV-induced mortality in mice (14). SV-induced death can also be blocked by the pluripotent anti-apoptotic protein, Bel-2 (8, 16). These data suggest that SV triggers a common death execution pathway, but the state changes induced by SV which set this common pathway in motion remain unclear.

Maintenance of the cellular redox balance is believed to play an important role in regulating survival (17), and multiple lines of evidence indicate that disruption of the redox equilibrium in favor of oxidants, a condition defined as “oxidative stress,” may activate apoptosis (18–20). In cultured cells, the addition of oxidants or inhibition of antioxidant defenses leads to apoptosis (18–20). Similarly, overexpression of antioxidant enzymes or treatment of cells with antioxidants has been shown to prevent apoptotic death (21–24). Apoptosis induced by a disruption in redox homeostasis appears to be a mechanism of neuronal loss in a broad range of sporadic and inherited neuropathological disorders, including Parkinson’s disease (25), Alzheimer’s disease (26), amyotrophic lateral sclerosis (27), and Huntington’s disease (28). Moreover, genetic studies indicate that inherited mutations in antioxidant defenses may also induce neuropathy (29, 30). For example, a subset of patients with a familial form of amyotrophic lateral sclerosis have been found to carry mutations in the gene encoding the antioxidant enzyme, copper/zinc superoxide dismutase (Cu,Zn-SOD) (29, 31, 32). Mutations in the Cu,Zn-SOD gene appear to diminish the threshold for activation of apoptosis in neurons, and antioxidants can restore the threshold for activation of cell death to levels seen in cells carrying wild type Cu,Zn-SOD (33). Taken together, these observations suggest that oxidative stress is a mediator of apoptotic death, although cell death induced by certain stimuli can be triggered independent of oxidative stress (34, 35).

To examine whether apoptosis induced by SV is activated by oxidative stress, we previously evaluated the effects of antioxidants on SV-induced apoptosis in cultured cells (36). Our results showed that a host of antioxidants, including metal chelators, inhibitors of lipid peroxidation, or scavengers of peroxide failed to inhibit SV-induced apoptosis, suggesting that oxidative stress is not a mediator of SV-induced death (36).
However, we did not monitor directly individual reactive oxygen species (ROS), such as superoxide (O$_2^-$) or peroxide (H$_2$O$_2$), so we could not definitively exclude a role for these ROS in regulating SV-induced death. In this study, we examine the role of O$_2^-$ and H$_2$O$_2$ in SV-induced apoptosis.

**MATERIALS AND METHODS**

**Cell Culture and Viability Studies—**AT-3 rat prostate carcinoma cells and 3T3 fibroblasts were cultured as described previously (36, 37). For viability studies, 10$^5$ AT-3 cells/well or 10$^4$ 3T3 fibroblasts/well were seeded in 96-well plates. 1 day after plating, recombinant SVs were added at a multiplicity of infection (m.o.i.) of 10 plaque-forming units per cell. Cell viability was determined by measuring the cell-associated lactate dehydrogenase activity as described previously (37). For the experiments involving pyruvate and paraquat (1,1’-dimethyl-4,4’-bipyridinium dichloride) (Sigma), AT-3 cells or 3T3 fibroblasts were pretreated with pyruvate or paraquat for 1 h and then infected with recombinant SVs. Viability assays were performed 24 h later as described above.

**Generation of Recombinant Virus—**A cDNA fragment containing Cu,Zn-SOD (SOD wt) or Cu,Zn-SOD with a glycine to serine mutation at codon 41 (SOD M) was cut from the PstI/EcoRI site of pTVL1392 and was then blunt end ligated into the BstEI site of an SV vector (dsTE1EQ) in forward and reverse orientations. A polymerase chain amplified DNA fragment containing catalase of *Hemophilus influenza* from pWB5 plasmid (a gift from Dr. William Bishai) (38) was also blunt end ligated into the BstEI site of a recombinant SV vector in forward and reverse orientations. These recombinant SV vectors were linearized with XhoI and then transcribed in vitro with SP6 RNA polymerase (Life Technologies, Inc.). The resultant RNA transcripts were transfected into baby hamster kidney cells by the LipofectAMINE method as described previously (37). Recombinant SVs were harvested from the supernatant of the culture media of the transfected baby hamster kidney cells. Viral titers were determined by standard plaque assays as described previously.

**Immunoblotting—**10$^5$ AT-3 cells were infected with recombinant SV-SODs (wt, M or reverse (R)) at an m.o.i. of 10. 1 h postinfection, cells were lysed as described previously (37). 20 μg of protein from cytoplasmic extracts was boiled in Laemmli buffer and electrophoresed under reducing conditions on 15% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad). Non-reducing conditions on 15% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride membrane (Bio-Rad). Non-specific binding was inhibited by incubation in Tris-buffered saline and Tween (50 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween 20) containing nonfat dried milk for 2 h. Human SOD antibody was diluted in 1% milk, Tris-buffered saline and Tween at 1:5,000. After exposure of membranes to horseradish peroxidase-conjugated secondary antibody for 1.5 h, immunoreactive proteins were detected according to the enhanced chemiluminescent protocol (Amersham Pharmacia Biotech).

**Aconitase Assay—**10$^5$ AT-3 cells or 3T3 fibroblasts were either infected with SV (AR339) at an m.o.i. of 25 or treated with paraquat. At various intervals after paraquat treatment or SV infection, cells were lightly trypsinized, pelleted, and resuspended in 100 μl of reaction mixture containing 50 mM Tris-HCl, pH 7.4, 30 mM sodium citrate, 0.6 mM MnCl$_2$, 0.2 mM NADP$,^+$, and 1 unit/ml isocitrate dehydrogenase (39–41). Cells were then homogenized by Tekmar Tissumizer for 10 s in the reaction mixture, and aconitase activity was then determined by the absorbance increase at 340 nm at 25 °C for 30 min in a Molecular Devices SpectraMax 250 thermostatted microplate UV-visible spectrophotometer. The results were expressed as (aconitase activity from each time point/aconitase activity from mock infected or mock treated cells) × 100%.

**SOD Activity Measurement—**SOD activity was determined by an indirect inhibition assay developed by Oberley and Spitz (42). Briefly, 4 × 10$^4$ AT-3 cells were either mock infected or infected with recombinant SV-SODs (wt, M, or R) at an m.o.i. of 10 for 16 h and then were trypsinized. Cell pellets were resuspended in 100 μl of potassium phosphate buffer (50 mM, pH 7.8) and then were homogenized by Tekmar Tissumizer for 1 min. 10 μg of cell homogenates was used for determining SOD activity in 200 μl of reagent containing 50 mM potassium phosphate buffer (pH 7.8), 5.6 × 10$^{-5}$ M nitro blue tetrazolium, 0.1 mM xanthine, and 0.002 unit of xanthine oxidase. The SOD activity of cell lysates was measured spectrophotometrically by monitoring the supression of superoxide-induced formation of formazan from nitro blue tetrazolium at 560 nm for 5 min.

**Catalase Activity Measurement—**Catalase activity was measured as described by Aebi (43). 4 × 10$^4$ AT-3 cells were mock infected or infected with recombinant SV-catalase (forward and reverse) for 20 h. Cell extracts were then prepared as described above. 10 μg of cell extracts was added to 100 μl of 10 mM H$_2$O$_2$ in 50 mM potassium phosphate buffer, pH 7.8, and the rate of loss of absorbance of H$_2$O$_2$ at 240 nm was monitored for 30 s in a Beckman DU-50 spectrophotometer.

**Sindbis Virus Production—**10$^5$ AT-3 cells or 3T3 fibroblasts were grown in 12-well plates and were infected with recombinant SV-SOD viruses at an m.o.i. of 10. 2 h after infection, cells were washed three times with phosphate-buffered saline, and then the infectious media were harvested at 4, 8, and 24 h postinfection. Virus titers were determined by standard plaque assay on baby hamster kidney cells. For paraquat experiments, 3T3 fibroblasts were treated with 250 μM paraquat 1 h prior to virus infection, and viabilities were assessed as described above at the indicated intervals.

**RESULTS**

**Infection with SV Reduces Intracellular Superoxide Levels in AT-3 Rat Prostate Carcinoma Cells—**Aconitase activity is a sensitive and specific assay for measuring intracellular O$_2^-$ levels in mammalian cells (44). To test whether O$_2^-$ levels change prior to SV-induced apoptosis, we assayed aconitase activity after SV (AR339) infection in AT-3 rat prostate carcinoma cells. Previous studies have established that SV (AR339) triggers cell death with morphological and biochemical features of apoptosis 18–24 h after infection in this cell line (8, 36). SV (AR339) increased aconitase activity within minutes of binding to and/or infecting AT-3 cells, suggesting that SV decreases O$_2^-$ prior to the onset of apoptosis (Fig. 1). To verify that aconitase activity is a valid measure of O$_2^-$ levels in AT-3 cells, we exposed these cells to paraquat, a redox cycling agent capable of generating O$_2^-$ intracellularly. As expected, we found that paraquat reduces aconitase activity beginning 5 min after drug exposure (Fig. 1).

**Recombinant SV Expressing Wild Type SOD (SV-SOD1 wt) Potentiates SV-induced Apoptosis—**To determine whether decreases in O$_2^-$ levels regulate SV-induced apoptosis, we generated a recombinant SV that carries the wild type human Cu,Zn-SOD gene (SV-SOD wt). As controls, we generated a mutated form of the human Cu,Zn-SOD gene (SV-SOD M) and the wild type human Cu,Zn-SOD gene in the reverse orientation (SV-SOD R), into the SV vector. SOD1 is a cytosolic enzyme that catalyzes the conversion of O$_2^-$ into H$_2$O$_2$. The mutated form of SOD1 used in this study has a glycine to serine mutation at codon 41. This mutation, which is predicted to disrupt the integrity of the β sheet initiated at codon 41 and the

**Fig. 1.** SV infection increases aconitase activity in AT-3 cells. AT-3 cells were infected with SV (AR339 (C)) at an m.o.i. of 25, and aconitase activity was determined at the indicated time points as described under “Materials and Methods.” In parallel, AT-3 cells treated with paraquat (○) were also harvested for aconitase activity assays. The percentage of aconitase activity was determined as (aconitase activity from each time point/aconitase activity from mock infected/treated cells) × 100%. Results represent the mean value from three to five independent experiments ± S.E.
adjoining active site loop containing the copper ion, was identified previously in a pedigree of familial amyotrophic lateral sclerosis patients and has been shown to cause a significant reduction in SOD1 activity in cultured lymphoblasts of affected patients compared with SOD1 activity in lymphoblasts from controls (32).

Infection of AT-3 cells with SV-SOD wt significantly enhances cell death compared with infection with recombinant SV-SOD M or recombinant SV-SOD R (Fig. 2A). The potentiating effect of wild type SOD1 on SV-induced apoptosis is also observed in 3T3 fibroblasts where 47, 70, or 65% of cells survive after infection with SV-SOD wt, SV-SOD M, or SV-SOD R, respectively (not shown).

SOD enzymatic activity was determined in lysates of mock, SV-SOD wt-, SV-SOD M-, or SV-SOD R-infected AT-3 cells. As expected, cells infected with SV-SOD wt significantly increased SOD enzymatic activity (1.7-fold) compared with mock infected cells. By contrast, SOD activity in AT-3 cells infected with each recombinant virus. SV-SOD wt, SV-SOD M, and SV-SOD R replicate with equal efficiency at early time points after infection and prior to the appearance of apoptotic morphology (Fig. 3). At 24 h postinfection, the virus production was slightly lower in cells infected with SV-SOD wt compared with virus production of cells infected with SV-SOD M and SV-SOD R. The difference in viral titers detected at later time points likely relates to the decreased viability of cells infected with SV-SOD wt (Fig. 3). Taken together, our results suggest that decreased intracellular O$_2^-$ levels enhance SV-induced apoptosis without affecting virus replication.

Inhibition of H$_2$O$_2$ Generation by Pyruvate Does Not Prevent the Potentiating Effect of Wild Type SOD on SV-induced Apoptosis—Enforced expression of wild type SOD has been shown to result in increased steady-state levels of hydrogen peroxide (H$_2$O$_2$) (45). Hydrogen peroxide can induce cytotoxicity by reacting with transition metals such as iron to form highly reactive hydroxyl radicals. To examine whether enhanced generation of H$_2$O$_2$ by wt SOD contributes to the death-promoting effect of recombinant SV-SOD wt, we treated 3T3 fibroblasts with pyruvate, a nonenzymatic scavenger of peroxide (46), 1 h prior to infection with recombinant SV-SOD wt, SV-SOD M, or SV-SOD R. Although 2 mM pyruvate completely inhibits cytotoxicity induced by 200 mM hydrogen peroxide in 3T3 (Fig. 4B) or AT-3 cells (not shown), pyruvate (2 mM or 10 mM) has no effect on the potentiation of SV-induced death by wild type SOD1 (Fig. 4A). These results suggest that potentiation of cell death by SV-SOD is not mediated through peroxide. However, we cannot exclude the possibility that SV induces metabolic barriers that prevent intracellular pyruvate from reaching a concentration required for peroxide scavenging.

To examine more definitively the role of H$_2$O$_2$ in SV-induced apoptosis, we generated a recombinant SV carrying a catalase gene from H. influenzae (SV-Cat.). Catalase is an antioxidant enzyme that catalyzes H$_2$O$_2$ into H$_2$O and O$_2$. As a control, we inserted catalase in the reverse orientation into the SV vector. Catalase activity measurements confirmed that infection of AT-3 cells with SV-Cat. resulted in increased levels of catalase activity compared with mock or SV-Cat. R-infected cells (Fig. 5A). To determine the effects of enhanced catalase activity on SV-induced apoptosis, we measured the viability of cells in-
levels and not increased or decreased scav-
which may inhibit compo-
but not peroxide in regulating SV-induced death. 
levels, leading to activation of cell death.
should

**FIG. 3.** Recombinant SV-SOD wt (■), SV-SOD M (○), and SV-
SOD R (△) replicate at similar rates in AT-3 cells. Supernatants
from AT-3 cells infected with recombinant SVs were collected at 4, 8, 
and 24 h postinfection, and plaque assay was performed in duplicate 
in baby hamster kidney cells. Results are expressed as the mean ± S.E. 
from three independent experiments. Error bars are hidden by the 
symbols.

**FIG. 4.** Pyruvate, a peroxide scavenger, does not reverse the 
potentiating effect of wild type SOD on SV-induced apoptosis in 
3T3 fibroblasts. Panel A, 3T3 fibroblasts were pretreated with 2 mM or 
10 mM pyruvate for 1 h and then infected with recombinant SV-SODs.
Cell viability was determined by lactate dehydrogenase assay at 24 h 
postinfection, and the results are presented as the mean ± S.E. from 
three independent experiments. A small and reproducible amount of 
cell death (<3%) was observed in mock infected cultures. Dotted bars, 
SV-SOD wt; gray bars, SV-SOD M; black bars, SV-SOD R. Panel B, 3T3 
fibroblasts were pretreated with 2 mM pyruvate for 1 h and then treated 
with 200 μM H2O2. Cell viability was determined after 5 h of H2O2 
treatment. Results are expressed as the mean ± S.E. from three 
experiments. Error bars are hidden by the symbols.

infected with various recombinant viruses at several time points 
after infection. As shown in Fig. 5B, we found that SV-Cat. and 
SV-Cat. R triggered cell death with similar kinetics in AT-3 cells.

Taken together, these results are consistent with the notion 
that the potentiating effects of wt SOD on SV-induced death 
relate to decreased O2 levels and not increased or decreased 
peroxide levels. Furthermore and consistent with our previous 
observations (36, 47), these data demonstrate that H2O2 is not 
a regulator of SV-induced apoptosis.

*Increasing Intracellular Superoxide by Paraquat Protects 
from SV-induced Apoptosis*—If decreases in superoxide trigger 
SV-induced death, then increases in intracellular O2 should 
abrogate SV cytotoxicity. To address this hypothesis, we 
treated 3T3 fibroblasts with 250 μM paraquat 1 h prior to 
infection with SV-SOD wt, SV-SOD M, and SV-SOD R. At 24 h 
postinfection, 250 μM paraquat significantly inhibits cell death 
induced by SV-SOD wt, SV-SOD M, and SV-SOD R (Fig. 6A).

To determine whether paraquat impairs SV replication, vi-
rus production from infected 3T3 fibroblasts with or without 
the treatment of paraquat was measured. Concentrations of 
paraquat which impair apoptosis do not interfere with virus 
replication at 4, 8, and 24 h postinfection (Fig. 6B).

**DISCUSSION**

Stable overexpression of individual antioxidant enzymes in 
cell lines has been shown to result in increased or decreased 
levels of other antioxidant enzymes in these lines (48, 49). For 
example, overexpression of SOD1 in NIH/3T3 cells has been 
shown to alter endogenous glutathione peroxidase, manganese 
superoxide dismutase, and glutathione transferase activities 
(48). Under these circumstances, the primary biological effects 
of modulating the levels of a single antioxidant enzyme may be 
difficult to evaluate. The recombinant SV expression system 
overcomes this problem by allowing the expression of heterol-
ogous proteins after infection, avoiding the need to generate 
stable cell lines and limiting the amount of time in which 
secondary changes can occur. Moreover, because recombinant 
SV can also induce apoptosis, the role of the expressed heter-
ologous proteins in regulating apoptosis can be evaluated (15, 16, 37). Herein, we used the recombinant SV system to over-
express SOD1 or catalase in AT-3 cells and to demonstrate a 
role for O2 but not peroxide in regulating SV-induced death.

Several observations suggest that SV infection leads to 
reduced intracellular O2 levels, leading to activation of cell death. 
First, aconitase activity, an *in situ* measure whose levels are 
inversed proportional to O2 levels (50), increases almost im-
mediately after SV infection. Second, expression of the O2 scav-
enger, SOD, in a recombinant SV vector, accelerates SV- 
duced apoptosis, whereas a mutated form of SOD, with 
impaired enzymatic activity, does not (Fig. 2A). Third, the O2 
generator, paraquat, inhibits SV-induced apoptosis (Fig. 6A).

These results suggest that intracellular O2 may inhibit compo-
nents of the death machinery required for SV-induced apopto-
sis. Of note, intracellular O2 has also been shown to be an 
endogenous inhibitor of Fas-induced apoptosis (51), suggesting 
that Fas- and SV-induced death may engage similar cell death 
pathways. Indeed, previous evidence supports the notion that 
Fas- and SV-induced apoptosis might activate a similar death 
pathway because both Fas- and SV-induced apoptosis are sen-
sitive to CrmA, a highly specific inhibitor of caspases 1, 4, 5, or 
8 (14).

Several lines of evidence indicate that Cu,Zn-SOD has a 
protective role in regulating apoptosis. For example, microin-
jection of Cu,Zn-SOD delays nerve growth factor deprivation-
induced apoptosis in sympathetic neurons (23). Also, overex-
pression of Cu,Zn-SOD inhibits apoptosis induced by serum or 
growth factor withdrawal or calcium ionophore treatment in 
nigral neural cells (52). Moreover, transgenic *Drosophila* that 
carry a mutated Cu,Zn-SOD gene appeared to have a shortened 
lifespan (53), whereas selective overexpression of the wild type 
Cu,Zn-SOD gene in motor neurons of *Drosophila* with defective
Cu,Zn-SOD recover 60% of their lifespan (54). In mammals, mice lacking Cu,Zn-SOD are more sensitive to axonal injury (55). These converging lines of inquiry suggest that Cu,Zn-SOD can promote survival in some circumstances. In contrast, transgenic mice overexpressing Cu,Zn-SOD appeared to have significantly fewer thymocytes and are more susceptible to lipopolysaccharide-mediated loss of thymocytes (56). Moreover, neurons derived from transgenic mice overexpressing Cu,Zn-SOD also showed higher susceptibility to kainic acid-induced apoptosis (57). Therefore, constitutive expression of a O2\textsuperscript{-} scavenging enzyme may also sensitize cells to certain kinds of stimuli; these observations are consistent with our observations that overexpression of Cu,Zn-SOD exacerbates SV-induced apoptosis.

In contrast to established schemes whereby ROS are increased prior to the activation of apoptosis (58), we find that intracellular ROS, specifically O2\textsuperscript{-} levels, are decreased soon after SV infection (Fig. 1). The mechanism by which SV triggers the decrease of intracellular O2\textsuperscript{-} is still unclear. However, this finding combined with our previous observation that SV infection leads to increased intracellular glutathione levels (36) suggests that SV may activate the apoptotic pathway by perturbing the redox equilibrium toward a reduced state rather than an oxidized state. Of note, a decreased production of ROS has also been shown to be an early event in dexamethasone-induced apoptosis in thymocytes (59), suggesting a more general role for “reductive stress” in activating cell death.

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What are the putative targets modified by decreased levels of intracellular O2\textsuperscript{-} which regulate SV-induced death? Our results suggest that decreasing intracellular O2\textsuperscript{-} does not facilitate SV replication because recombinant SV-SOD1 wt replicates as well as the SV-SOD1 M and SV-SOD1 R (Fig. 3). Additionally,
paraquat protects from SV-induced apoptosis and does not alter SV replication (Fig. 6B). Therefore, it seems likely that O$_2^-$ may play a role in changing the activity of as yet unidentified cellular targets involved in regulating SV-induced apoptosis. O$_2^-$ itself is capable of inactivating certain kinases, such as mammalian creatine phosphokinase (60) and the NADH dehydrogenase complex of the mitochondrial electron transport chain (61); however, the roles of these proteins in apoptosis are still unknown. Another potential target of O$_2^-$ are the caspase- cysteine-dependent proteases whose activity has been shown to be altered by redox-active agents (62). It is unlikely that the target modulated by superoxide levels which negatively regulates SV-induced cell death is aconitase or its iron-deficient analog IRP-1, as previous studies from our laboratory showed that the iron chelators do not inhibit SV-induced death in AT3 cells (36). If decreased IRP-1 activity or increased aconitase activity were the mechanism by which the apoptotic signal is mediated, then iron chelators, which enhance IRP-1 activity and decrease aconitase activity (63), should attenuate SV-induced death.

In addition to O$_2^-$, nitric oxide has also been shown to inhibit SV-induced apoptosis in cultured cells and diminish SV-mediated mortality in mice (64). Treatment of fetal mice with a nitric oxide synthase inhibitor potentiates SV-induced encephalitis and mortality; in addition, treatment of N18 neuroblastoma cells with nitric acid donors enhances cell survival after SV infection. These results combined with our observations suggest that O$_2^-$ and nitric oxide are the free radical species that negatively regulate SV-induced apoptosis.

Previously, we have demonstrated that SV-induced apoptosis may not result from the generation of ROS, as a panel of antioxidants failed to protect from SV-induced apoptosis (36). Here, in an extension of our previous study, we demonstrate that inhibition of intracellular O$_2^-$ levels is required for SV-induced apoptosis. These results not only provide insight into the mechanism by which SV transduces its death signal but also broaden the concept of oxidative stress-induced apoptosis; that is, reductive stress may also trigger cells to undergo apoptosis.

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