Nutrition- and Virus-Induced Stress Represses the Expression of Manganese Superoxide Dismutase *in Vitro*

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The relationship between oxidative stress and neuronal cell death has been suggested for many years. To understand the influence of oxidative stress on neuronal cell death, we investigated the influence of oxidative stress on DEV cells, a human glial cell line. Using enterovirus infection and/or malnutrition to induce oxidative stress, our results demonstrate that those stressors severely influence the antioxidant defense system in DEV cells. Although the expression of mitochondrial manganese superoxide dismutase (MnSOD) in DEV cells was significantly increased in acute infection with viral and nutritional stress, in persistent infection and nutritional stress, the expression of the MnSOD was drastically downregulated. We believe that this downregulation of MnSOD expression in the chronic stress model is due to repression of antioxidant defense. The downregulation of the MnSOD expression may lead to an increase of free-radical production and thus explain why the cells in the chronic stress model were more vulnerable to other oxidative stress influences. The vulnerability of DEV cells to additional stress factors resulted in progressive cell death, which may be analogous to the cell death in neurodegenerative diseases. Exp Biol Med 229:843–849, 2004

Key words: *in vitro* stress model; oxidative stress; persistent virus infection; enteroviruses

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Oxygen free-radicals have been implicated as a cause of neuronal death in neurodegenerative diseases such as in Parkinson disease (1–3), Alzheimer disease (4, 5), and amyotrophic lateral sclerosis (6). This hypothesis became more accepted when it was demonstrated that antioxidant treatment can ameliorate or attenuate the progression of neurodegenerative diseases (7). In addition, there is increasing evidence that there is a link between oxidative stress and mitochondrial dysfunction (8, 9). Because mitochondrial metabolism is the principal source of high-energy and free-radical intermediates, a decline in function may lead to an increase in oxidative stress, impaired calcium buffering, activation of the mitochondrial permeability transition, and secondary excitotoxicity (10).

To protect the cells from oxygen free-radicals, the mammalian cells have an enzymatic and nonenzymatic scavenging system. Antioxidant enzymes such as superoxide dismutase, catalase, and gluthathione peroxidase are enzymes that remove O_2^- and H_2O_2 from the tissue (11). Superoxide dimutase (SOD) activity is especially important for the antioxidant defense. There are two SOD enzyme classes depending on the metals contained. A superoxide dismutase containing copper and zinc is located in the cytoplasm and nucleus, whereas a manganese-containing superoxide dismutase is located predominantly in the mitochondria. Because mitochondria are the main source for free-radical production (12), the MnSOD is the most important antioxidant defense enzyme. Although the MnSOD is increased during oxidative stress and free-radical production, there are also circumstances in which oxidative stress can lead to a decrease of the MnSOD. Such a decrease in MnSOD is found in certain cancers (13), early and late stages of HIV infection (14), and during prion infection (15). Why the MnSOD is decreased during these few circumstances is not clear, as there are many more examples of increased MnSOD during free-radical formation. A nuclear DNA mutation in mice inactivates the mitochondrial

MnSOD and results in death within 8 days (16). In addition, partial MnSOD deficiency chronically increases oxidative stress, decreases oxidative phosphorylation function, and stimulates apoptosis (17, 18). However, the association between oxidative stress, mitochondrial dysfunction, and neuronal cell loss is not well understood.

To determine how stress influences the mitochondrial antioxidant defense system MnSOD, we used acute and chronic *in vitro* stress models. A human glial cell line, DEV, was exposed to acute and chronic stress induced by viruses and/or malnutrition. Malnutrition as a stressor may increase the susceptibility of the human host to a variety of disease agents; for example, enterovirus infection, which is reflected in Keshan disease (19), or the neuropathy outbreak in Cuba (20). By using malnutrition and enterovirus infection as stresses in acute and chronic *in vitro* stress models, we demonstrated that extreme stress can lead to a decrease of MnSOD. In contrast, moderate stress upregulated the MnSOD protein expression. To our knowledge, this is the first *in vitro* model that demonstrates the decrease of MnSOD during the continuous stress of a chronic enterovirus infection and malnutrition.

Materials and Methods

Viruses. Echovirus 6 prototype "d'Amori" (EV6), an enterovirus from the family of Picornaviridae, was obtained from the National Reference Center of Enteroviruses (Lyon, France). EV6 is a common lytic enterovirus that can be associated with acute neuromeningitic syndromes and can also persist in human cells (21).

Cells. DEV cells, a primary human glial cell line that retains the capacity to differentiate toward astrocytes (22) and shares many characteristics with primary neural stem cells (23), were obtained from Dr. Giraudon (Faculté de Médicine, Lyon, France). DEV cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Cergy Pontoise, France) containing 10% fetal calf serum (FCS) (Gibco) and cultured in 25 cm^2 tissue culture flasks (T25 flasks) (Falcon, Le Pont de Claix, France).

Virus Infection. DEV cells were trypsinized and incubated in DMEM with 10% FCS at 37°C in T25 flasks. When the monolayer was 70% confluent, the cells were washed once with phosphate-buffered saline (PBS) (Life Technology, Cergy Pontoise, France) followed by the addition of 2 ml DMEM containing desired dose of EV6 virus. After an incubation period of 1.5 hrs at 37°C, the cells were washed with PBS, and 5 ml DMED containing 10% FCS (10% FCS-DMEM) was added.

Virus Titration. MRC 5 cells (10^4 cells/well) were seeded in 96-well microtiter plates and incubated at 37°C to obtain a full monolayer. Twenty-five microliters from each 10-fold dilution of virus (10^{-1} to 10^{-8}) was added to 5 wells each. Cells were maintained in 2% FCS-DMEM at 37°C for 5 days when the virus titer as mean tissue culture infective dose ($TCID_{50}$) was calculated following the method of Reed and Muench (24).

Stress Induction. *Acute Stress Model.* Acute virus infection and/or nutrition depletion (DMEM without FCS) were used to induce stress reactions in DEV cells. For the acute stress models with viruses, DEV cells were infected with EV6 at an inoculum dosage of 0.1 $TCID_{50}$/cell in a T25 flask with 70% cell confluence. The cells were collected at 30 mins, 2, 4, 6, 20, and 30 hrs after infection.

For acute stress with nutritional depletion, DMEM without FCS was initiated either 10 days before or immediately after the 1.5-hr viral adsorption period. In either case, the nutritional depletion continued up to 10 days after infection. Controls included uninfected and non-nutritionally stressed DEV cells.

Chronic Stress Model. To establish a chronic stress model using persistent viral infection, very low innoculum of EV6 at a multiplicity of 0.001 $TCID_{50}$/cell was used to infect DEV cells in a T25 flask. When a cytopathic effect was observed (2 to 3 days after infection), the cells were washed once with PBS and incubated in DMEM with 10% FCS. When the remaining EV6 infected cells established a confluent monolayer, the cells were trypsinized again. With subsequent serial cell passages (every 2 to 3 weeks), the presence of EV6 was determined by titration of both supernatant and the cell pellet onto MRC-5 cells.

To establish nutritional stress, confluent monolayers of cells 1.5 months after infection were separated into 2 groups. In one group, cells were grown in DMEM with 10% FCS, and in the other group, cells were grown with DMEM without FCS establishing nutritional stress. For analysis, cells were collected before nutritional stress and from Day 1 to Day 8 during nutritional stress.

Measurement of Cell Viability: MTT Cell Proliferation Assay. The MTT test is a colorimetric assay system that measures the viability of cells by the reduction of tetrazolium (MTT) into an insoluble formazan product by their mitochondrial enzyme succinate dehydrogenase (Roche Diagnostic, Meylan, France).

To test the cell viability in our chronic stress model, uninfected and EV6 infected cells were distributed at 10^4 cells per well to 96-ell plates. After 0 to 8 days during nutritional stress induction, 10 µl of the MTT labeling reagent was added to each well and incubated at 37°C for 4 hrs. Then, 100 µl of solubilization solution was added per well and incubated at 37°C overnight. The results were analyzed with an enzyme linked immunosorbent assay (ELISA) plate reader at a wavelength of 550 nm and a reference wavelength of >650 nm. Results are expressed as the average of three different experiments.

Western Blotting. MnSOD proteins were obtained alternatively from uninfected or infected DEV cells, lysed in 10 vol lysis buffer (1% triton X and 1 tablet of the Complete protease inhibitor cocktail [Roche] in 50 ml Tris-buffered saline, pH 7.8). The concentration of the proteins was determined with the Bio-Rad DC Protein assay as described

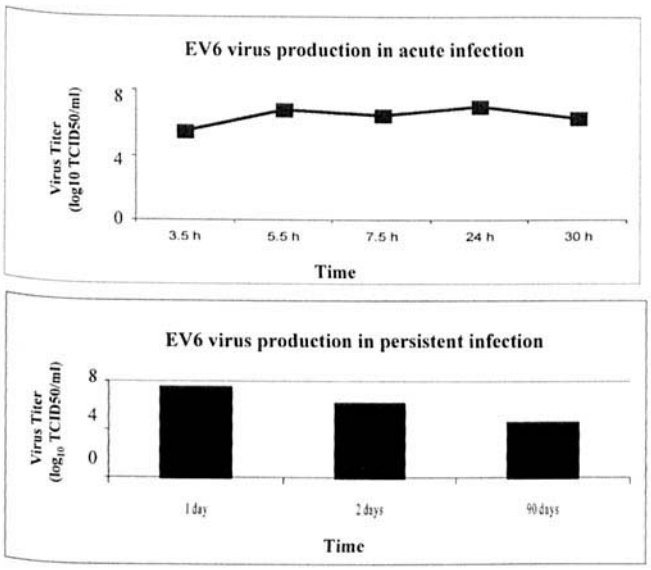

Figure 1. Variation of echovirus 6 prototype "d'Amori" (EV6) virus production during acute (30 hrs) or persistent (90 days) infection of DEV cells. The virus titer is expressed as the logarithm$_{10}$ $TCID_{50}$.

by the manufacturer (Bio-Rad Laboratories, Hercules, CA). Five to 10 μg of proteins were loaded on a 15% sodium dodecyl sulfate–polyacrylamide gel and transferred to a nitrocellulose membrane at 15 V for 30 mins with the Trans Blot semi-dry cell (BioRad). Membranes were blocked with 5% dry milk in PBS, pH 7.4, for 1 hr at 37°C. Anti-MnSOD antibody (1:500) was incubated with the membranes overnight at 4°C. After rinsing the membranes 3 times with PBS containing 0.1% Tween 20 and 1% dry milk, rabbit anti-human IgG conjugated with horseradish peroxidase (1:5000 dilution) was applied. Following 1 hr incubation at 37°C, membranes were washed 4 times and developed with the ECL Western blotting detection reagents (Amersham Pharmacia Biotech, York, UK).

Results

Acute and Chronic Stress Established with Virus Infection. To investigate the effect of stress on the MnSOD protein expression, both an acute and a chronic stress model were established using virus infection as the stress factor. DEV cells were infected with EV6 at multiplicity of 0.1 $TCID_{50}$/cell in a T25 flask when there was 70% cell confluence. The plateau of viral titer in the culture medium was reached by 5.5 hrs of infection, with a concentration approaching 10^7 $TCID_{50}$ (Fig. 1). These acutely infected cells died within 48 hrs after infection. The MnSOD was measured 2, 4, 6, 8, 12, 20, and 30 hrs after infection.

To investigate the impact of continuous stress on the MnSOD protein expression, a chronic stress model was established using persistent virus infection. DEV cells in a T25 flask with a 70% cell confluence were infected with EV6 at a multiplicity of 0.001 $TCID_{50}$/cell in a T25 flask. The viral titer in the EV6 infected cells reached a peak of 3 $\times$ 10^7 $TCID_{50}$ after 1 day (Fig. 1). Although 80% of these cells died within 72 hrs after infection, the remaining 20% of the cells survived and recovered. The recovered cell morphology appeared normal and could be passed as easily as uninfected control cells. These cells were persistently infected with EV6 virus, however, and virus could be demonstrated easily. The virus titer in the culture medium was between 10^4 and 10^5 $TCID_{50}$ throughout the experiment up to 90 days (Fig. 1). The MnSOD protein expression was measured after 90 days.

The Mitochondrial Antioxidant Response in an Acute Stress Model. The acute stress model was used to determine if a short induction of stress due to virus or nutritional stress, or both in combination, can modulate the MnSOD protein expression. Interestingly, neither stress factor was able to upregulate the MnSOD protein expression significantly, alone, or in combination during the first 2 days of stress induction (Fig. 2a). All the cells were viable up to 20 hrs (Fig. 2b).

In a second assay, DEV cells were nutritionally stressed for 10 days before they were infected with EV6. After 10 days of nutritional stress alone, the MnSOD protein expression was increased significantly in these cells (Fig. 3a). However, additional virus infection on these nutritionally stressed cells had no significant influence on the MnSOD protein expression. Although control and nutritionally stressed cells had enhanced viability at 30 hrs, the viability of the EV6 infected cells was unchanged, and the viability of the cells both infected and nutritionally stressed were slightly reduced (Fig. 3b). The nutritionally stressed cells were able to survive at least for 3 weeks (Table 1). If FCS was added to the cells shortly before cell death, these nutritionally stressed cells could recover and were able to grow again.

The Mitochondrial Antioxidant Response in a Chronic Stress Model. DEV cells were infected with EV6 for 90 days and then nutritionally stressed. The MnSOD protein expression and cell proliferation were measured by Western blot and the MTT proliferation assay, respectively. Nutritional stress alone induced the MnSOD protein expression significantly at 8 days. The stress of persistent EV6 infection alone showed no increase in MnSOD protein expression when compared to unstressed cells. The addition of nutritional stress to the stress of persistent EV6 infection downregulated the MnSOD protein expression drastically below the level of detection by Western blot. These cells died within 8 days (Fig. 4b), and addition of fresh nutrition did not prevent death. Both cells persistently infected with EV6 and uninfected control cells could survive more than 40 cell passages (Table 1). Cells depleted by nutrition alone survived up to 3 weeks and could be recovered when FCS was added to the media.

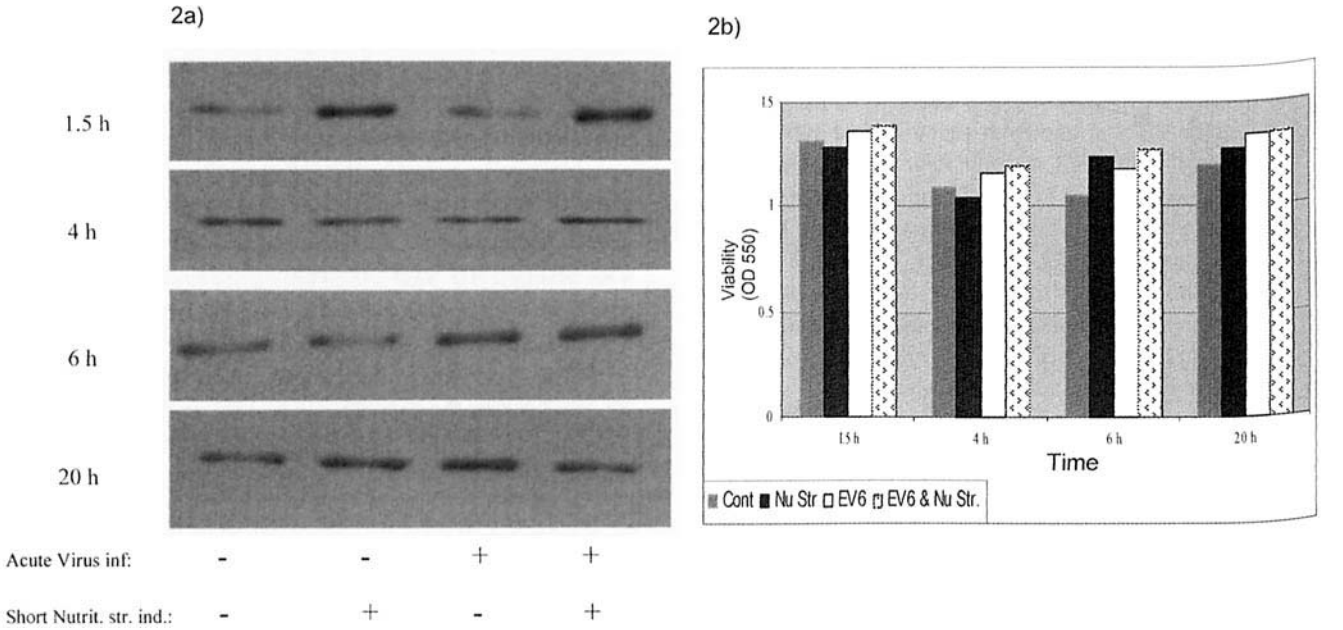

Figure 2. (a) MnSOD protein expression over 20 hrs by DEV cells that are or are not acutely infected with echovirus 6 prototype "d'Amori" (EV6) and are or are not exposed to short nutritional stress beginning 1.5 hrs after virus infection. (b) Viability measured by using the MTT test of DEV cells that have been acutely infected with EV6 and are or are not exposed to nutritional stress (inf., infection; nutrit. str. ind., nutritional stress induction; Cont, control; Nu. Str., nutritional stress).

Discussion

The results obtained from the current study using an *in vitro* acute and chronic stress model support the *in vivo* evidence that stress can influence the induction of MnSOD, the major antioxidant defense system in mitochondria. Contrary to the acute stress model where the MnSOD was augmented as predicted, in the chronic stress model, the MnSOD was unexpectedly decreased.

The alteration of the antioxidant enzymes by oxidative stress has been demonstrated by others (25). It is also well-known that neurons and other brain cells are continually challenged by conditions that may cause acute and chronic

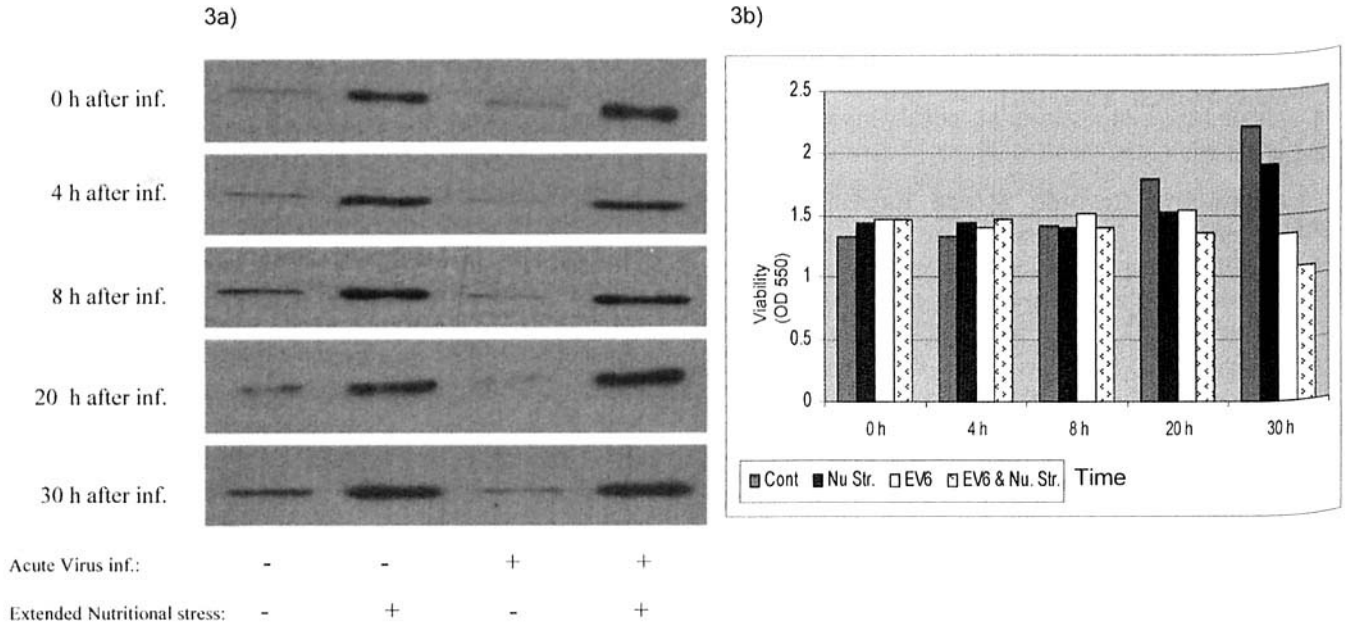

Figure 3. (a) MnSOD protein expression over 30 hrs of infection of DEV cells that are or are not acutely infected with echovirus 6 prototype "d'Amori" (EV6) and are or are not exposed to extensive nutritional stress beginning 10 days prior to infection. (b) Viability using the MTT test of DEV cells that have been acutely infected with EV6 and are or are not exposed to nutrional stress beginning 10 days prior to infection (inf., infection; nutrit. str. ind., nutritional stress induction; Cont, control; Nu. Str., nutritional stress).

Table 1. Viability of DEV Cells After Induction of Different Stressors[a]

Stress factors	DEV cell viability
None	~10 months (>40 passages)
EV6 persistent infection	~10 months (>40 passages)
Nutritional stress	~3 weeks
EV6 persistent infection and nutritional stress	~7 days

[a] DEV, human glial cell line; EV6, echovirus 6 prototype "d'Amori."

stress and could result in oxidative stress. To protect the brain from these extracellular factors, there are a variety of antioxidant defenses, including enzymes, to protect against the oxygen free-radicals. However MnSOD, the mitochondrial enzyme, is one of the primary proteins involved in the cellular defense against oxidative stress. The decrease of MnSOD seen in our chronic model is especially noteworthy, as mitochondria are the chief producers of oxygen free-radicals. This decrease may lead to an elevation of reactive oxygen species and thus to a further impairment of mitochondrial function.

Mitochondrial dysfunction and oxidative stress have been connected with neuronal cell death in some degenerative diseases such as in Huntington disease, Parkinson disease, Alzheimer disease, and amyotrophic lateral sclerosis and also in prion disease (26–30).

Surprisingly, in prion disease, the activities of other antioxidant defense enzymes in the brain such as cytosolic CuZn SOD and catalase are not altered, whereas the mitochondrial MnSOD expression is downregulated. It has been suggested that the decreased activity of MnSOD might result in increasing oxidative stress in the mitochondria of the infected brain. It has also been suggested that mitochondria dysfunction caused by oxidative stress may give rise to the neurodegeneration in prion disease (15). MnSOD downregulation has also been described in other diseases. Flores *et al.* (31) found that in early and late HIV infection, there is a decrease of MnSOD in both blood plasma and mononuclear cells, including lymphocytes. It has been suggested that this is due to the HIV tat protein, which downregulates the synthesis and overrides the induction of the MnSOD (14).

MnSOD downregulation has also been described in cancer. In cancer cells, it has been assumed that the reduced MnSOD level was probably due to defective expression of the gene rather than a defect in the primary structure of MnSOD protein or a deletion of the MnSOD gene (13).

The decrease of MnSOD in our chronic stress model is due to the combination of a persistent enterovirus infection and nutritional stress. In this study, stress response in neuronal-derived cells were induced due to serum depravation. The nutritional components of serum for cell growth can be growth factors, hormones, or other factors. Although the current data did not point to any particular factor that

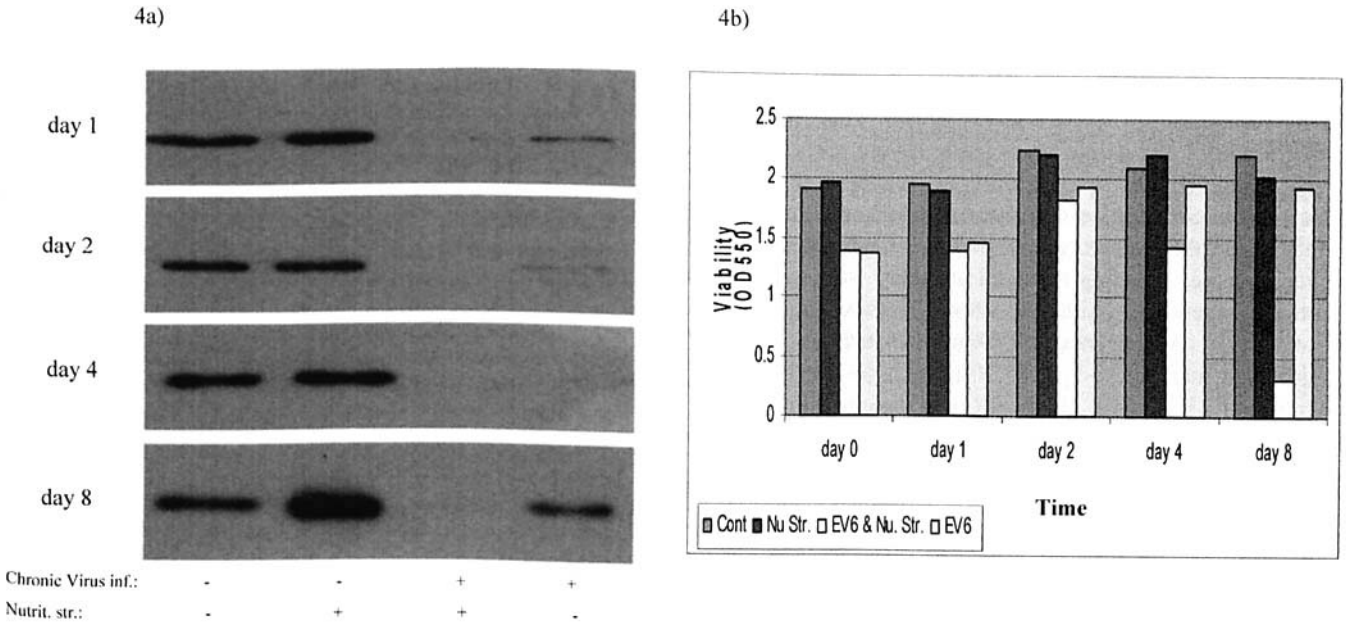

Figure 4. (a) MnSOD protein expression over 8 days by DEV cells that are or are not persistently infected for 90 days with echovirus 6 prototype "d'Amori" (EV6) and are or are not exposed to nutritional stress. (b) MTT results of the viability over 8 days of DEV cells that have or

may be the sole response for the stress response induction, multiple factors may be involved in supporting the growth of this neuronal-derived cell; depravation of any of the essential factors may cause cell stress response.

That the combination of nutritional stress and enterovirus infection can lead to disease has already been demonstrated in Keshan disease (32). Moreover, Beck *et al.* (33) confirmed in a mouse model that nutritional stress exacerbates the pathogenesis of Cox B3 infection. In addition, patients with acute coxsackievirus myocarditis had an increased level of free oxygen radicals and decreased level of antioxidants (34). Furthermore, it appears that some viruses (e.g., influenza virus) have mechanisms to control the cellular oxidant status. Influenza virus can increase the production of superoxide by lung epithelial cells due, in part, to increased activity of the superoxide generating enzyme xanthine oxidase (35). Oxidative stress is thought to be involved in the progression of HIV-induced disease, because O_2^- significantly enhances cell-to-cell transmission of HIV-1 (36). During persistent hepatitis B virus (HBV) infection in humans, DNA strand break induced by oxidative stress may increase HBV DNA integration events (37). Although the above examples demonstrate that oxidative stress may be necessary to increase virus replication or disease progression, it is still not known what may contribute to the downregulation of MnSOD or how downregulation of MnSOD expression is advantageous.

In the current *in vitro* report, infected cells under nutritional stress had downregulation of MnSOD and died earlier than cells that were only nutritionally stressed or only virus infected. In fact, the cells chronically infected with echovirus 6 survived over months and cells only nutritionally stressed survived weeks.

These results are comparable with the results from Copin *et al.* 2000 (38) who reported that mice expressing only 50% of the normal complement of MnSOD increased their susceptibility to oxidative stress. This increased susceptibility to oxidative stress led to severe mitochondrial dysfunction. Higher susceptibility to oxidative stress was also seen in SID2tmCje null mice that lacked a functional MnSOD. In these mice, mitochondrial enzyme deficiency, severe disease, and early neonatal mortality were associated with mitochondrial oxidative stress. If these mice were treated with an exogenous SOD mimic such as catalytic antioxidant manganese-5,10,15,20-tetrakis (4-benzoic acid) porphyrin (MnTBAP), they still had progressive neurodegenerative changes in the cortical and subcortical regions that were characterized by marked mitochondrial swelling and disorganization of cristae. The treated mice developed a progressive movement disorder (39). That the early mortality or mitochondrial impairment was due to oxygen free-radicals has been supported by the yeast model of Van Loon *et al.*, 1986 (40). They inactivated the MnSOD in yeast, and incubated the mutants with varying concentrations of oxygen. Increasing concentrations of oxygen inhibited the growth of these mutants.

In summary, we have demonstrated that acute and chronic stress due to viral infection and malnutrition can markedly influence the MnSOD of the mitochondrial antioxidant defense system. The mitochondrial MnSOD was severely downregulated in cells chronically infected with enterovirus if exposed to nutritional stress. On the other hand, nutritional stress to cells acutely infected with enteroviruses upregulated MnSOD. We believe that an excess of stress factors may lead to downregulation of MnSOD, which further increases the formation of free radicals, oxidative stress, and cell death. The mechanism for this downregulation is not clear at this time. The chronic stress model, however, provides the possibility of studying the mechanism of MnSOD downregulation. Such studies might be useful in understanding the downregulation of the expression of MnSOD protein that occurs in certain neurodegenerative diseases. To our knowledge, this is the first report that demonstrates a downregulation of MnSOD *in vitro* due to chronic enterovirus infection and malnutrition.

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