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NAD^+ treatment decreases tumor cell survival by inducing oxidative stress

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1. ABSTRACT

NAD^+ plays important roles in various biological processes. It has been shown that NAD^+ treatment can decrease genotoxic agent-induced death of primary neuronal and astrocyte cultures, and NAD^+ administration can reduce ischemic brain damage. However, the effects of NAD^+ treatment on tumor cell survival are unknown. In this study we found that treatment of NAD^+ at concentrations from 10 microMole to 1 mMole can significantly decrease the survival of various types of tumor cells such as C6 glioma cells. In contrast, NAD^+ treatment did not impair the survival of primary astrocyte cultures. Our study has also indicated that oxidative stress mediates the effects of NAD^+ on the survival of tumor cells, and P2X7 receptors and altered calcium homeostasis are involved in the effects of NAD^+ on the cell survival. Collectively, our study has provided the first evidence that NAD^+ treatment can decrease the survival of tumor cells by such mechanisms as inducing oxidative stress. Because NAD^+ treatment can selectively decrease the survival of tumor cells, NAD^+ may become a novel agent for treating cancer.

2. INTRODUCTION

A large number of studies have indicated that NAD^+ plays important roles in not only energy metabolism and mitochondrial functions, but also aging, gene expression, calcium homeostasis and immune functions (1-3). It has also been found that NAD^+ is a cytoprotective agent for primary cultures of astrocytes, neurons and myocytes: The death of these types of cells induced by various insults, including oxidative stress (4), DNA alkylating agents (5), oxygen-glucose deprivation (6) and zinc (7), can be profoundly decreased by NAD^+ treatment. In contrast, it has been found that NAD^+ can induce death of $CD4^+CD25^+$ T regulatory cells (Treg cells) (8, 9): The mono(ADP-ribose) transferases on the plasma membranes can consume NAD^+ to produce mono(ADP-ribosyl)ation of P2X7 receptors, which can promote the activation of the receptors leading to cell death.

Because cancer is one of the most lethal diseases affecting a large population, it is of great theoretical and clinical significance to find novel approaches to kill cancer cells and to elucidate new mechanisms underlying cancer

cell death. Because the effects of NAD^+ on tumor cell survival remain unknown, in this study we studied the effects of NAD^+ on the survival of various types of tumor cells. Our study has found that NAD^+ can profoundly decrease the survival of tumor cells by such pathways as generating oxidative stress.

3. MATERIALS AND METHODS

3.1. Materials

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO) except where otherwise noted.

3.2. Cell Cultures

C6 glioma cells, Neuro-2a cells, MKN-45 cells, and MCF-7 cells were purchased from the Cell Resource Center of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. The cells were plated in 24-well cell culture plates at an initial density of 1×10^5 cells/ml in Dulbecco's Modified Eagle Medium (containing 4,500 mg/L D-glucose, 584 mg/L L-glutamine, 110 mg/L sodium pyruvate) (Thermo Scientific, Waltham, MA, USA) that contains 1% penicillin and streptomycin (GIBCO BRL, Grand Island, NY, USA) and 10% fetal bovine serum (PAA, Linz, Austria). The cells were used when the densities of the cell cultures reached 60-80%.

3.3. Experimental Procedures

Experiments were initiated by replacing the culture medium with the culture medium with various concentrations of drugs. The cells were left in the incubator with 5% CO_2 at 37 ^oC for 24 or 48 hrs.

3.4. Lactate Dehydrogenase (LDH) Assay

As described previously (10), cell survival was quantified by measuring LDH activity in cell lysates. In brief, cells were lysed for 20 min in lysing buffer containing 0.04% Triton-X100, 2 mM HEPES, 0.2 mM dithiothreitol, 0.01% bovine serum albumin, and 0.1% phenol red, pH 7.5. Fifty μl cell lysates were mixed with 150 μl 500 mM potassium phosphate buffer (pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate, and the A_{340nm} change was monitored over 90 s. Percentage cell survival was calculated by normalizing the LDH values of the drug-treated samples to the LDH values of control (wash only) culture wells.

3.5. Trypan Blue Assay

After NAD^+ treatment, the incubation media was collected so as to collect the cells that were not attached to the bottom of the wells. Subsequently, trypsin was added into the wells for collections of the cells that were attached to the bottom of the wells. The Trypan blue-negative cells in both of these two collected cell populations were analyzed by Trypan blue assay by using a Beckman Coulter Vi-cell^{TM}XR Cell Viability Analyzer.

3.6. Flow Cytometry-Based PI Staining

Cells were digested with 0.1% trypsin and resuspended in 1 ml PBS. After washes with PBS twice, the cells were incubated with propidium iodide (20 μg/ml) at 37 ^oC for 30 min. Subsequently the number of PI-negative and PI-positive cells was assessed by a BD flow cytometer.

3.7. Dihydroethidium (DHE) Assay

After treatment, the cell cultures were incubated with 5 μM DHE for 30 min at 37 ^oC. Subsequently the cells were washed once with PBS, and the fluorescence signals were observed under a Leica fluorescence microscope at excitation wavelength of 545 nm and emission wavelength of 605 nm.

3.8. Statistical Analyses

All data are presented as mean $\pm$ SE. Data were assessed by one-way ANOVA, followed by Student-Newman-Keuls *post hoc* test. *P* values less than 0.05 were considered statistically significant.

4. RESULTS

4.1. NAD^+ treatment decreased the survival of various types of tumor cells

To determine the effects of NAD^+ treatment on the survival of tumor cells, we applied LDH assay, Trypan blue assay and flow cytometry-based PI assay. The LDH assays indicated that NAD^+ treatment for 24 or 48 hrs significantly decreased the number of surviving C6 glioma cells (Figure 1A), Neuro-2a cells (Figure 1B), MKN-45 cells (Figure 1C), and MCF-7 cells (Figure 1D): NAD^+ at concentrations of 10 – 1000 μM decreased by 10-80% the survival of the cells. For MKN-45 cells (Figure 1C), and MCF-7 cells (Figure 1D), treatment of the cells with as low as 1 μM NAD^+ significantly decreased the survival of the cells. Our Trypan blue assays also showed that NAD^+ treatment for 24 or 48 hrs significantly decreased the number of surviving C6 glioma cells (Figure 2A). Our flow cytometry-based PI assays further showed that NAD^+ treatment led to significantly reduced number of PI-negative C6 glioma cells (Figure 2B), indicating that NAD^+ treatment decreased the survival of the cells. In contrast, we found that treatment of primary rat astrocytes with 1 mM NAD^+ for up to 48 hrs did not affect the survival of the cells (data not shown).

4.2. Oxidative stress mediates the effects of NAD^+ on C6 glioma cells

Oxidative stress is one of the major inducers of cell death under many conditions. Therefore, we tested our hypothesis that NAD^+ decreases the tumor cell survival by generating oxidative stress. By using DHE assay that detects superoxide levels in the cells, we found that treatment of the cells with NAD^+ for 24 hrs induced increases in the red fluorescence signals, indicating increased generation of superoxide (Figure 3). We further found that treatment of the cells with two different types of antioxidants, including Trolox (Figure 4A) and N-acetyl cysteine (Figure 4B), significantly attenuated the effects of NAD^+ on the cell survival.

4.3. P2X7 receptors and altered calcium homeostasis are involved in the effects of NAD^+ on tumor cell survival

Previous studies have suggested that P2X7 receptors mediate NAD^+-induced death of T regulatory

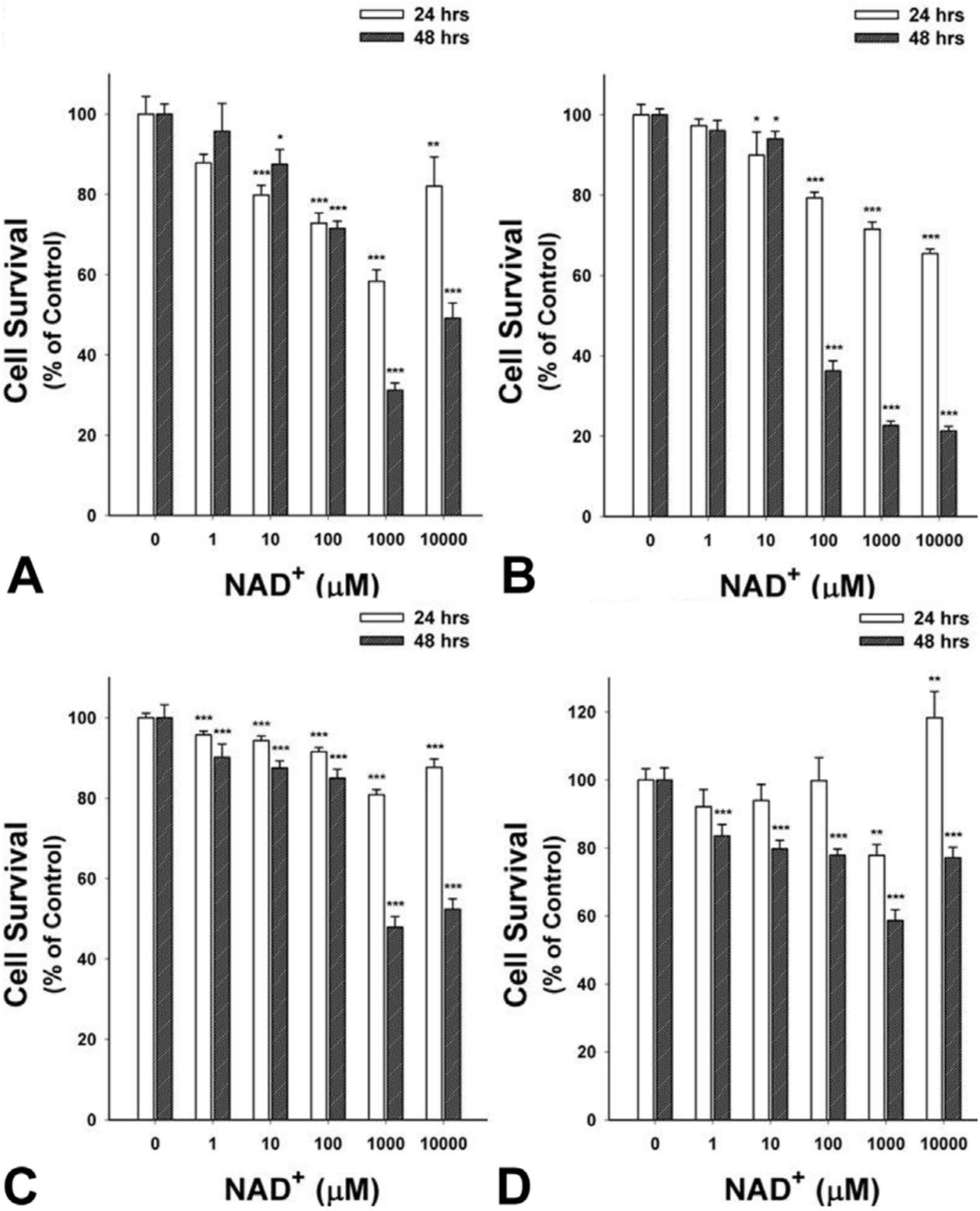

Figure 1. NAD^+ treatment decreased the survival of various types of tumor cells, as assessed by LDH assay. C6 glioma cells (A), Neuro-2a cells (B), MKN-45 cells (C), and MCF-7 cells (D) were treated with 1, 10, 100, 1000 or 10000 μM NAD^+ for 24 or 48 hrs, and subsequently cell survival was assessed by LDH assay. N = 10-29. Data were pooled from 3-7 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

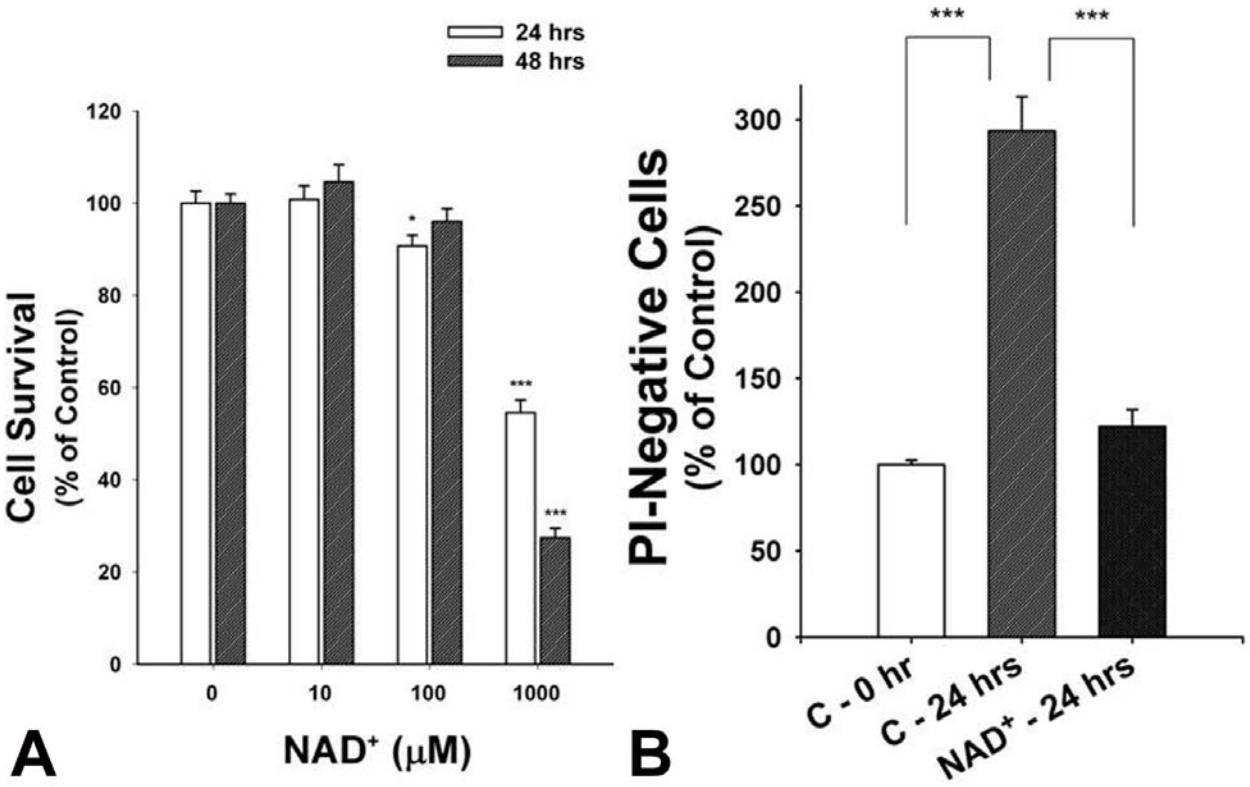

Figure 2. (A) NAD^+ treatment decreased the survival of C6 glioma cells, as assessed by Trypan blue assay. C6 glioma cells were treated with 10, 100 or 1000 μM NAD^+ for 24 or 48 hrs, and subsequently the number of surviving cells was assessed by Trypan blue assay. N = 16. Data were pooled from 4 independent experiments. *, $p < 0.05$; ***$p < 0.001$. (B) NAD^+ treatment decreased the survival of C6 glioma cells, as assessed by flow cytometry-based PI staining assay. C6 glioma cells were treated with 1000 μM NAD^+ for 24 hrs, and subsequently the number of surviving cells was assessed by flow cytometry-based PI staining assay. N = 14. Data were pooled from 5 independent experiments. ***, $p < 0.001$.

cells (8, 9). We determined if P2X7 receptors may also contribute to the NAD^+-induced decreases in the tumor cell survival by using C6 glioma cells as a cell model. We found that the effects of NAD^+ on the glioma cells were partially prevented by treatment of the cells with the broad spectrum P2X receptor antagonist pyridoxal-phosphate-6-azophenyl-2′,4′-disulfonate [PPADS](11) (Figure 5A), and blocked by treatment of the cells with the selective P2X7 receptor antagonist oxidized ATP (oxATP) (12) (Figure 5B). By applying the Ca^{2+} chelator BAPTA-AM, we also obtained evidence suggesting a role of altered calcium homeostasis in the effects of NAD^+ on C6 glioma cells: The NAD^+-induced decreases in the survival of C6 glioma cells were partially prevented by treatment of the cells with BAPTA-AM (Figure 6).

4.4. Nicotinamide treatment did not affect the survival of C6 glioma cells

Nicotinamide is a major degradation product of NAD^+, which has been shown to decrease cell death induced by such insults as oxidative stress (13-15). To determine if NAD^+ may affect the survival of the tumor cells by producing nicotinamide, we assessed the effects of nicotinamide on the survival of C6 glioma cells. Nicotinamide at concentrations up to 1 mM did not affect the survival of the tumor cells (Figure 7).

5. DISCUSSION

The key findings of this study include: First, NAD^+ treatment can markedly decrease the survival of various types of tumor cells, suggesting that NAD^+ is a novel agent for decreasing tumor cell survival; second, oxidative stress mediates the effects of NAD^+ on the survival of tumor cells; third, P2X7 receptors and altered calcium homeostasis are involved in the effects of NAD^+ on the tumor cell survival; and fourth, NAD^+ does not appear to affect tumor cell survival by generating its degradation product --- nicotinamide.

Increasing evidence has indicated that NAD^+ plays critical roles in a variety of biological functions, including energy metabolism, mitochondrial functions, aging, gene expression, calcium homeostasis and immune functions (1-3). A number of studies have also indicated that NAD^+ treatment is highly protective against of the toxicity of primary cultures of astrocytes, neurons and myocytes, which was induced by various insults such as oxidative stress (4-7). Our previous studies have also indicated that NAD^+ administration can be used to decrease ischemic brain damage (16). Thus, NAD^+ has become an increasingly promising cytoprotective agent (17). However, it has also been indicated that NAD^+ can induce

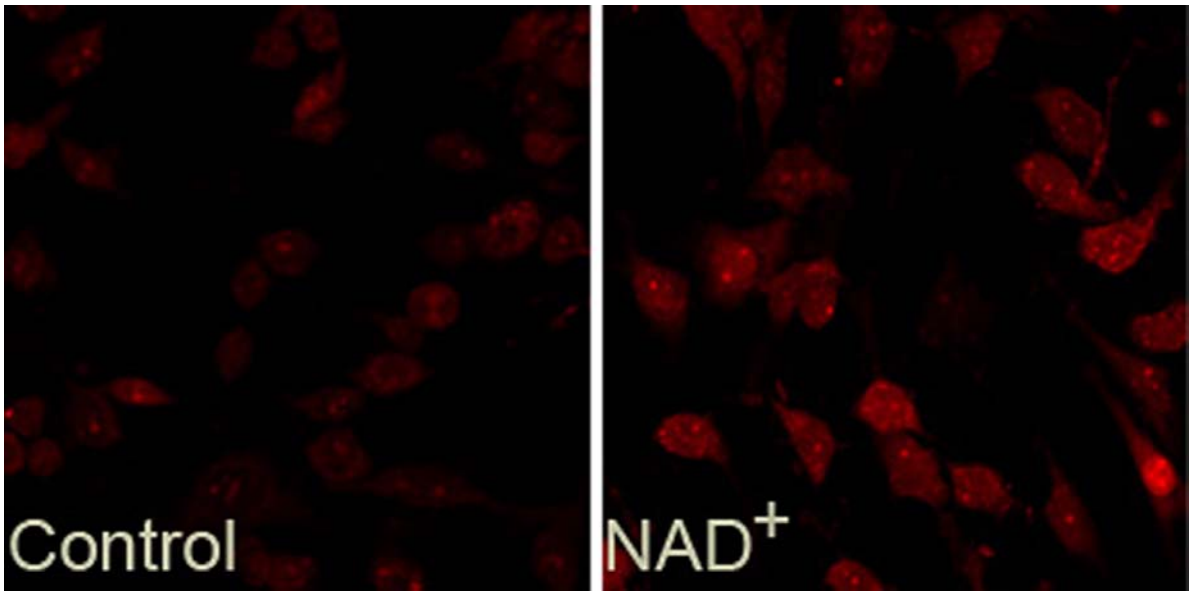

Figure 3. NAD^+ treatment increases the levels of ROS in C6 glioma cells. The cells were treated with 1 mM NAD^+ for 24 hrs, subsequently the ROS levels in the cells were determined by dihydroethidium (DHE) assay. Photos are representatives of 3 independent experiments.

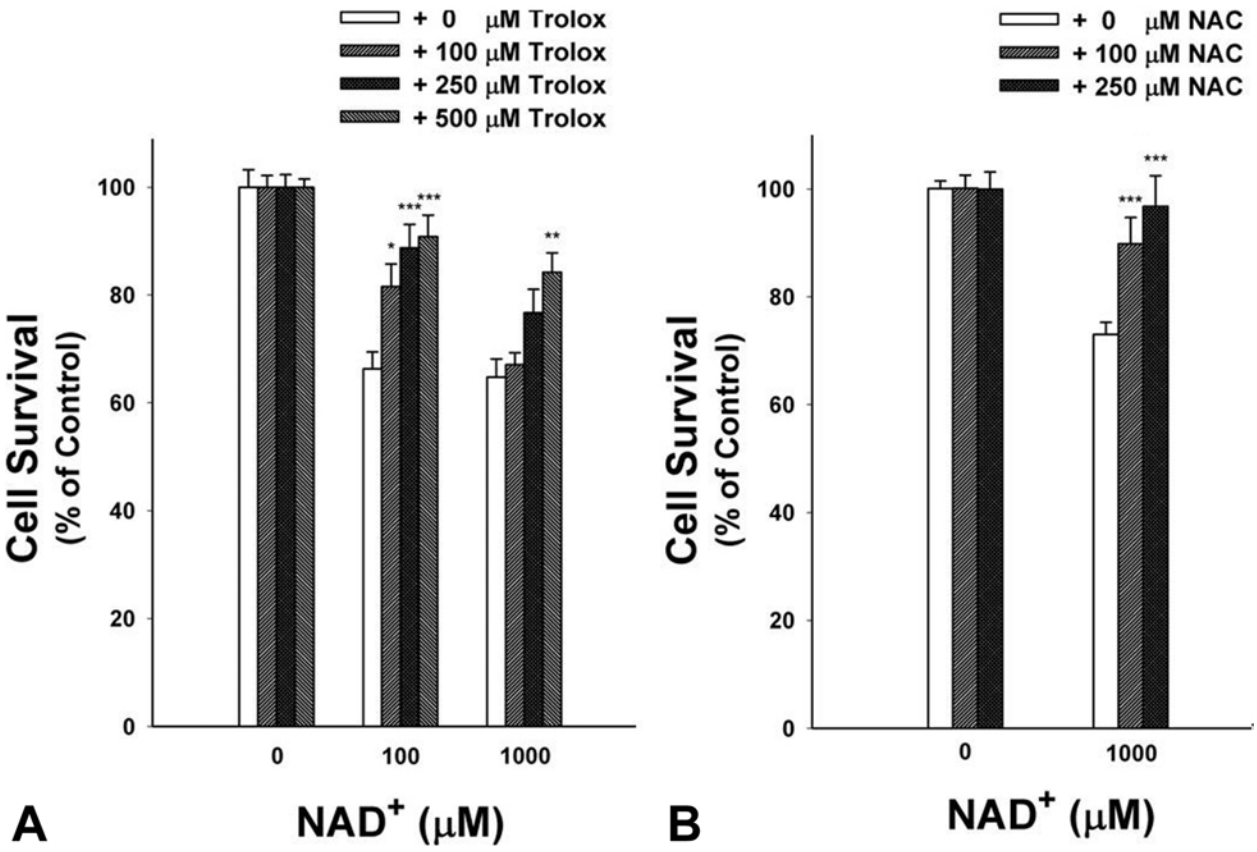

Figure 4. Antioxidants attenuated the effects of NAD^+ on the survival of C6 glioma cells. The cells were pre-treated with Trolox (A), or N-acetyl cysteine (B) for 30 min, followed by co-treatment with 0.1 or 1 mM NAD^+ for 24 hrs. The percentage of cell survival was assessed by LDH assay. N = 9-34. Data were pooled from 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

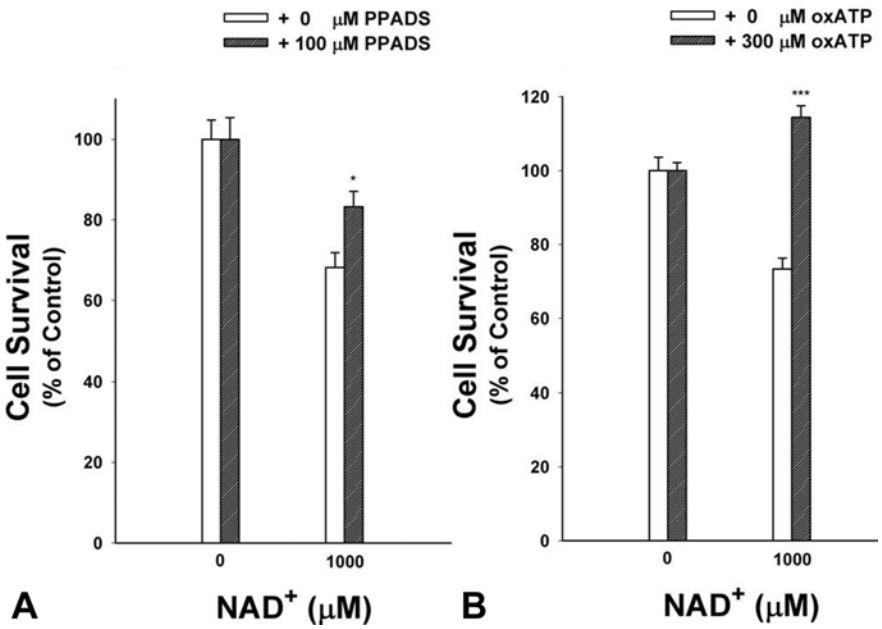

Figure 5. Evidence suggesting a significant role of P2X7 receptors in the NAD^+-induced decreases in C6 glioma cell survival. (A) The cells were pre-treated with [PPADS] a broad spectrum antagonist of P2X receptor for 30 min, followed by co-treatment with 1 mM NAD^+ for 24 hrs. The percentage of cell survival was assessed by LDH assay. (B) The cells were pre-treated oxATP --- a selective P2X7 receptor antagonist --- for 3 hrs. After washout of oxATP, the cells were treated with 1 mM NAD^+ for 24 hrs. The percentage of cell survival was assessed by LDH assay. N = 12-20. Data were pooled from 3 independent experiments. *, $p < 0.05$; ***, $p < 0.001$.

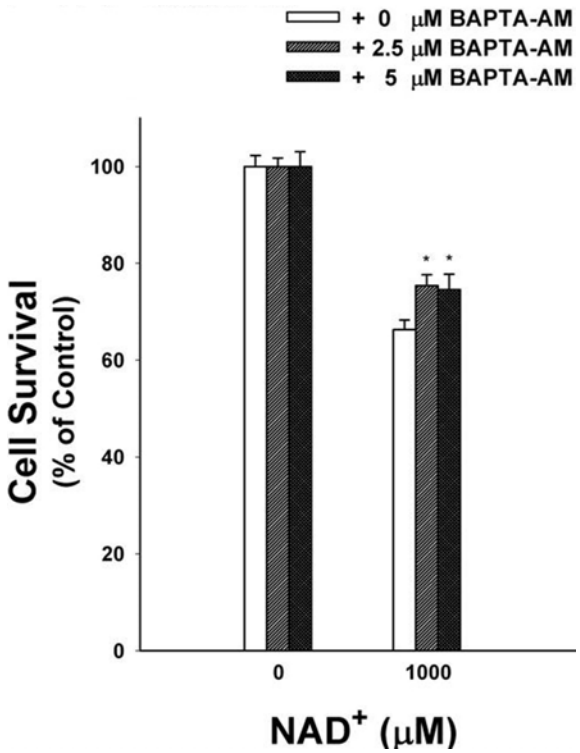

Figure 6. Evidence suggesting a role of altered calcium homeostasis in NAD^+-induced decreases in C6 glioma cell survival. The cells were pre-treated with the Ca^{2+} chelator BAPTA-AM for 60 min, followed by co-treatment with 1 mM NAD^+ for 24 hrs. The percentage of cell survival was assessed by LDH assay. N = 17-27. Data were pooled from 3 independent experiments. *, $p < 0.05$.

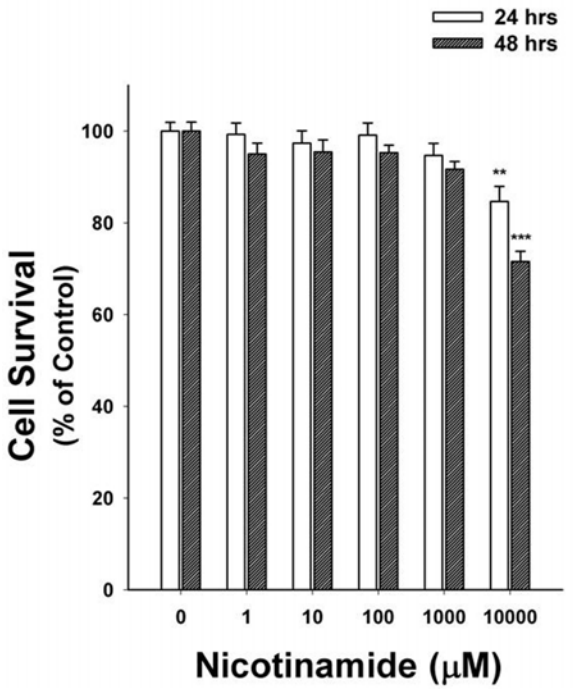

Figure 7. Nicotinamide --- the degradation product of NAD^+ --- at concentrations up to 1 mM did not affect the survival of C6 glioma cells. N = 11-23. Data were pooled from 3 independent experiments.

death of Treg cells (8, 9) by promoting mono(ADP-ribosyl)ation of P2X7 receptors, leading to activation of the receptors and subsequent cell death.

There has been no previous information regarding the effects of NAD^+ treatment on tumor cell survival. Our current study, by applying LDH assay, Trypan blue assay and flow cytometry-based PI assay, has provided the first evidence demonstrating that NAD^+ can significantly decrease the survival of various types of tumor cells. Interestingly, as low as 10 μM NAD^+ can produce statistically significant decreases in the survival of several types of tumor cells, suggesting that the effects of NAD^+ on tumor cell survival can be achieved by relatively low concentrations of NAD^+. Our study has strongly indicated that oxidative stress mediates the effect of NAD^+ on the cell survival: As shown by the DHE assay, NAD^+ can induce marked increases in ROS in the cells at 24 hours after the treatment; and two structurally different antioxidants --- Trolox and N-acetyl cysteine --- can significantly attenuate the effects of NAD^+. It is not surprising that ROS plays a significant role in the effects of NAD^+ on tumor cell survival, since oxidative stress is one of the major cell death-inducing factors under a variety of physiological and pathological conditions (18-20).

Our study has also suggested that another protein on the plasma membrane --- P2X7 receptors --- is involved in the effects of NAD^+ on the tumor cell survival, since both [PPADS] a broad-spectrum antagonist of the receptor, and oxATP --- a selective antagonist of the receptor, can attenuate the effects of NAD^+ on tumor cell survival. A number of studies have indicated important roles of P2X7R in various biological processes, including apoptosis, inflammation and pain (21). It has also been indicated that NAD^+ induces death of certain population of T regulatory cells by promoting the mono(ADP-ribosyl)ation of P2X7 receptors thus enhancing the activation of the receptors (8, 9). In our study, it remains unclear how NAD^+ may affect the P2X7 receptor activation. It is warranted to investigate the mechanisms underlying the roles of P2X7 receptors in the effects of NAD^+ on the tumor cell survival. One of the key effects of P2X7 receptor opening is increased influx of Ca^{2+} into cells (22). Thus, our observation that P2X7 receptors are involved in the effects of NAD^+ on the cell survival has implicated that NAD^+ may induce increased calcium influx into the cells. Indeed, our study applying the calcium chelator BAPTA-AM has indicated that BAPTA-AM is capable of decreasing the effects of NAD^+, suggesting a significant role of altered calcium homeostasis in the effects of NAD^+ on the cell survival.

Nicotinamide is a major degradation product of NAD^+, which has been shown to decrease cell death induced by such insults as oxidative stress (13-15). Our study has provided evidence arguing against the possibility that NAD^+ induces the necrosis of cancer cells by producing nicotinamide: In contrast to the significant cell loss induced by 10 – 100 μM NAD^+, nicotinamide at even 1 mM did not affect the cell survival.

A number of studies from our laboratory and other groups have shown that NAD^+ can protect primary cultures of astrocytes, neurons and myocytes against various insults (4-7, 23). These previous studies, together with our current study, have suggested that NAD^+ selectively kills tumor cells, while it can produce great protective effects for primary cell cultures against various insults. Cancer has remained to be one of the most lethal and threatening diseases for human being. While a number of drugs have been designed for treating cancer, nearly all of the cancer cell-killing drugs can produce toxic side effects on normal tissues. Our study suggests that NAD^+ may become an exceptional cancer cell-killing drug, due to its unique characteristics that it selectively kills cancer cells while being highly protective for normal cells.

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