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NADPH treatment decreases C6 glioma cell survival by increasing oxidative stress

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1. ABSTRACT

NADPH (nicotinamide adenine dinucleotide phosphate, reduced form) plays pivotal roles in antioxidation and reductive biosynthesis. However, the effect of NADPH treatment on cell survival is unknown. In this study, we determined the effect of NADPH treatment on the survival of glioma cells. Treatment of C6 glioma cells with as low as 1 μM NADPH for 24 hrs induced a significant decrease in the survival of the glioma cells, while NADPH treatment had no effect on the survival of primary astrocyte cultures. We also found that NADPH treatment increased intracellular oxidative stress. Three antioxidants and the NADPH oxidase inhibitor, apocynin, attenuated the effect of NADPH. Poly(ADP-ribose) polymerase (PARP) activation appears to be a downstream effector of the oxidative stress, since PARP inhibitors reduced the effect of NADPH. Calcium chelator, BAPTA-AM, also attenuated the effect of NADPH. Collectively, these data indicate a novel property of NADPH: NADPH decreases glioma cell survival by inducing the NADPH oxidase-dependent increase in oxidative stress and by PARP activation. These results also suggest a potential therapeutic effect of NADPH on gliomas.

2. INTRODUCTION

NADPH is a key molecule for cellular antioxidation, which is required for regeneration of GSH (1-3). NADPH also plays significant roles in reductive biosynthesis of nucleic acid and fatty acid (1-3). Seeming paradoxically, NADPH is also required for reactive oxygen species (ROS) generation by NADPH oxidase, which increasingly appears to be a critical source of ROS (4-6). While numerous studies regarding the biological functions of NADPH have been conducted, there has been little information regarding the effects of NADPH treatment on cellular functions and cell survival.

Cancer is one of the most devastating diseases affecting a large population. Therefore, it is of great significance to find novel approaches to kill cancer cells and to elucidate new mechanisms underlying cancer cell death. Because the effects of NADPH treatment on cell survival remain unknown, in this study we determined the effects of NADPH on the survival of C6 glioma cells and searched for the potential mechanisms underlying the influences of NADPH on the tumor cell survival. Because NADPH oxidase is a key enzyme for generating oxidative

stress (3-5), NADPH may promote NADPH oxidase-dependent generation of ROS, which is a key factor in both necrosis and apoptosis (7, 8). Thus, NADPH treatment may induce cell death by promoting NADPH oxidase-dependent ROS generation. Based on these pieces of information, in this study we also determined the roles of NADPH oxidase and ROS in the potential effects of NADPH on tumor cell survival.

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 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 the 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 (Invitrogen, Carlsbad, CA, 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. Lactate dehydrogenase (LDH) assay

As described previously (9), 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 X-100, 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 samples to LDH activity measured in lysates from control (wash only) culture wells.

3.4. Determinations of nuclear condensation

The nuclear size of cells was assessed by Hoechst staining (10). In brief, cells were treated with 20 μg/ml Hoechst 33258 in phosphate buffered saline (PBS) for 20 min. The stained nuclei were photographed under a fluorescence microscope. To quantify the size of the nuclei, three randomly picked fields in each well were photographed.

3.5. Flow cytometry-based PI staining

C6 glioma cells (70-100% confluence) 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 ^{o}C for 30 min. Subsequently the number of PI-negative and PI-positive cells was assessed by a flow cytometer (BD FACSAriaII).

3.6. Trypan blue Assay

After drug 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.

3.7. Dihydroethidium (DHE) assay

The cell culture were incubated with 5 μM DHE for 30 min at 37 ^{o}C. 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. NADPH treatment can dose-dependently decrease the survival of C6 glioma cells

To assess the effects of NADPH treatment on the survival of C6 glioma cells, we applied both LDH assay and Trypan blue assay. Our LDH assay showed that NADPH treatment dose-dependently decreased the number of surviving C6 glioma cells at 24 hrs after NADPH treatment (Figure 1A): NADPH at concentrations of 1 – 1000 μM decreased by 10-40% the survival of the glioma cells. Similar results were obtained by applying Trypan blue assay (Figure 1B).

Our flow cytometry studies also showed that the number of NADPH-treated cells was markedly lower compared with the cell number of controls (Figure 2A). The flow cytometry-based PI assay of the cells showed that NADPH induced significant increases in PI-stained cells (Figures 2B and 2C), suggesting that NADPH can induce cell necrosis. We further determined the effect of long-term treatment of C6 glioma cells with various concentrations (1, 10, 100 and 1000 μM) of NADPH: Treatment of the cells with 1 mM NADH for 4 days decreased by nearly 90% the survival of the cells (Figure 3). In contrast, we found that treatment of primary rat astrocytes with 1 mM NADPH for up to 48 hrs did not affect the survival of the cells (data not shown).

We studied if NADPH treatment may induce nuclear condensation of the cells --- a hallmarker of cell apoptosis. We found that NADPH treatment did not produce nuclear condensation of the cells (data not shown). Instead, 1 mM NADPH induced an increase in the nuclear size of the cells, arguing against the possibility that NADPH affects cell survival by inducing apoptosis.

4.2. Oxidative stress mediates the effects of NADPH on C6 glioma cell survival

Since oxidative stress is one of the major inducers of cell death under many conditions (7, 8), we tested our hypothesis that NADPH induces glioma cell

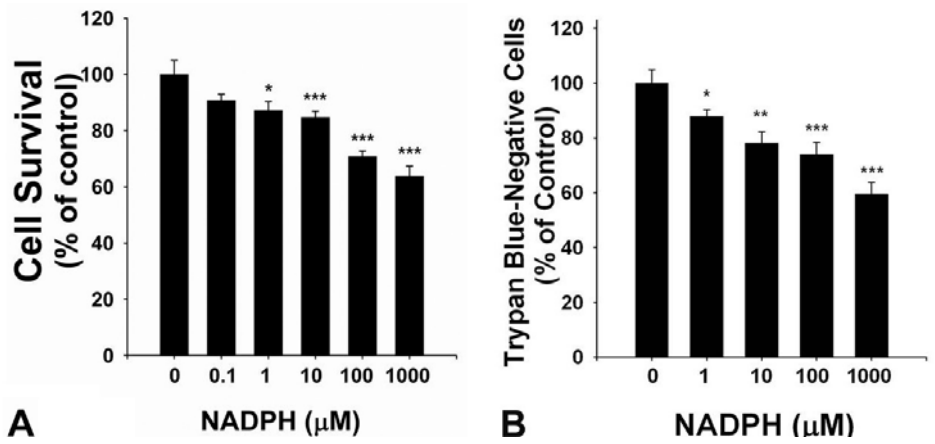

Figure 1. NADPH treatment dose-dependently decreased the number of surviving C6 glioma cells at 24 hrs after NADPH treatment, as assessed by LDH assay (A) or Trypan blue assay (B). The cells were treated with 0.1, 1, 10, 100 and 1000 μM NADPH for 24 hrs, and subsequently the number of surviving cells was assessed by LDH assay or Trypan blue assay. N = 12-13. Data were pooled from 3-4 independent experiments. *□$p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

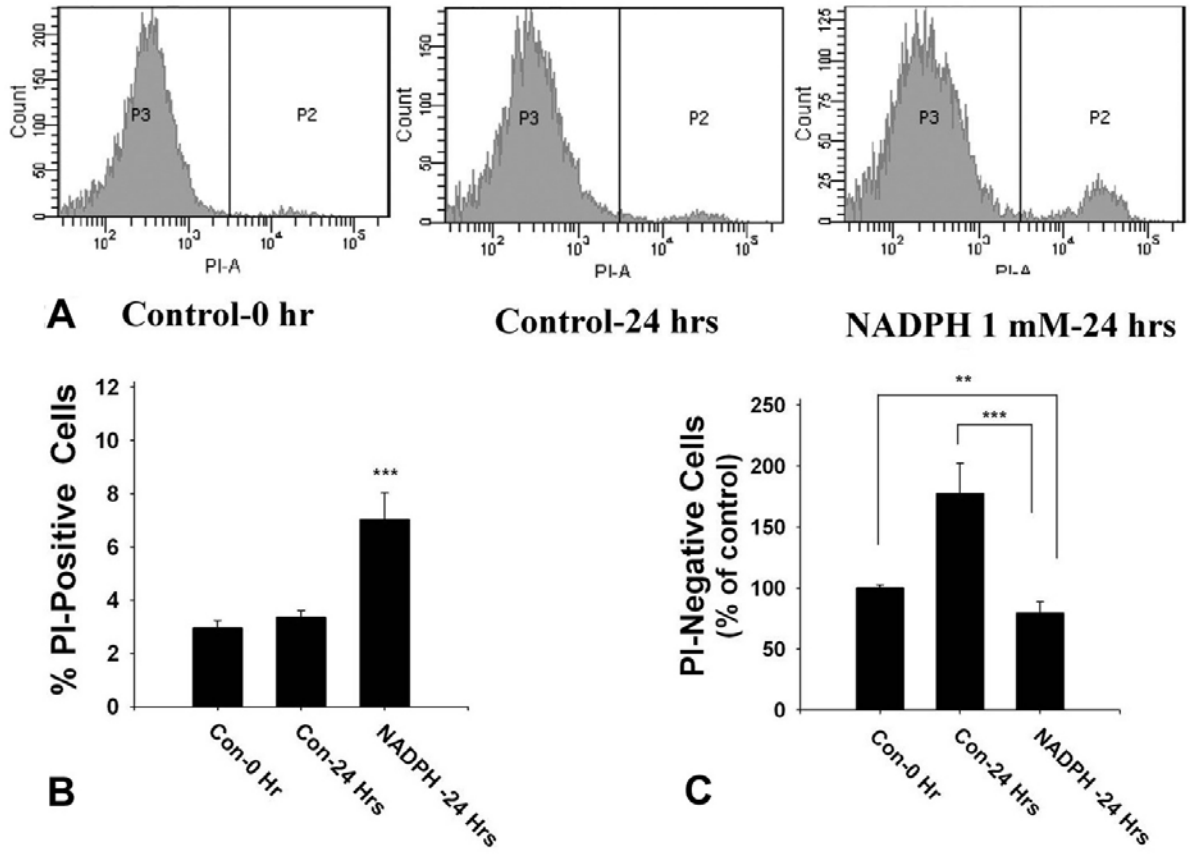

Figure 2. (A) Quantifications of the results from the flow cytometry-based PI assays showed that NADPH treatment induced a significant decrease in the number of PI-negative C6 glioma cells. (B) The graphs from flow cytometry-based PI assays showed that NADPH treatment induced an increase in the number of PI-positive C6 glioma cells: The P2 fraction and the P3 fraction indicate the number of PI-positive cells and PI-negative cells, respectively. The number of PI-positive cells in the samples treated with 1 mM NADPH appeared to be greater than that in the controls. (C) Quantifications of the results from the flow cytometry-based PI assays showed that NADPH treatment induced a significant increase in the number of PI-positive C6 glioma cells. The cells were treated with 1 mM NADPH for 24 hrs, and subsequently the number of PI-positive cells was assessed by flow cytometry-based PI assay. N = 8-16. Data were pooled from 3-4 independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

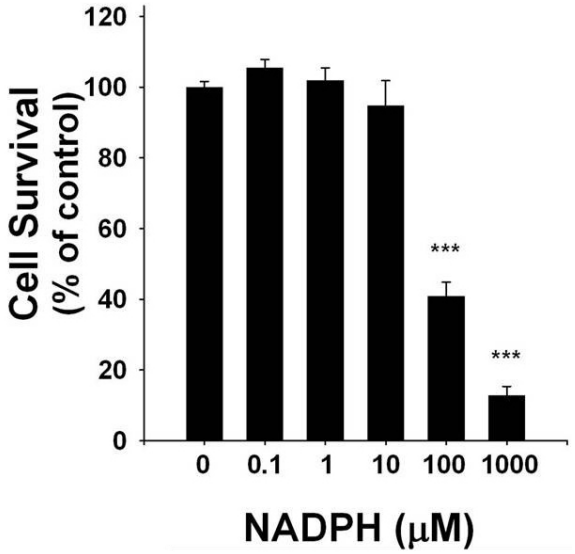

Figure 3. NADPH treatment for 4 days profoundly decreased the number of surviving C6 glioma cells. The cells were treated with 0.1, 1, 10, 100 and 1000 μM NADPH for 4 days, and subsequently the number of surviving cells was assessed by LDH assay. N = 20. Data were pooled from 3 independent experiments. ***, $p < 0.001$.

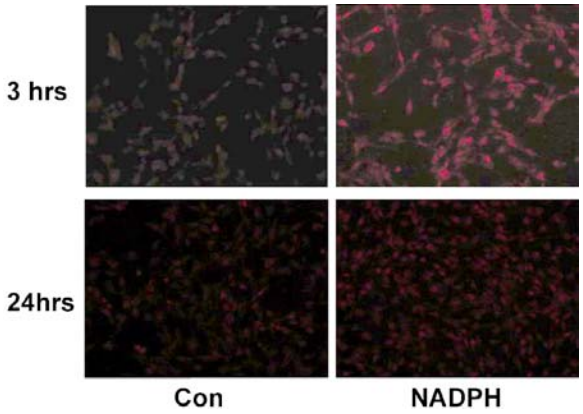

Figure 4. NADPH treatment increases the levels of ROS in C6 glioma cells. The cells were treated with 1 mM NADPH for 3 or 24 hrs, subsequently the ROS levels in the cells were determined by dihydroethidium (DHE) assay. Photos are representatives of 4 - 6 independent experiments.

death by generating oxidative stress. By using DHE assay that detects superoxide levels in the cells, we found that treatment of the cells with NADPH for 3 hrs or 24 hrs induced increases in the red fluorescence signals, indicating increased generation of superoxide (Figure 4). We further found that treatment of the cells with three different types of antioxidants, including Trolox (Figure 5A), Tempol (Figure 5B), and N-acetyl cysteine (Figure 5C), significantly attenuated the effects of NADPH on the cell survival.

4.3. NADPH oxidase and PARP play significant roles in the effects of NADPH on C6 glioma cell survival

Since NADPH oxidase is a major ROS-generating enzyme, which generates ROS by using NADPH as the substrate (3-5), we tested if NADPH oxidase is responsible for the effects of NADPH on the cell survival. We found that the NADPH oxidase inhibitor apocynin significantly decreased the effects of NADPH on C6 glioma cells (Figure 6), suggesting a significant role of the enzyme in the effects of NADPH on the cell survival. PARP is a key enzyme in oxidative stress-induced cell death (3, 11, 12). We tested if PARP activation is also involved in this effect of NADPH on the cell survival. The NADPH-induced decreases in C6 glioma cell survival were partially prevented by treatment of the PARP inhibitors (3,4-dihydro-5-(4-(1-piperidinyl)butoxy)-1(2H)-isoquinolinone) (DPQ) (Figure 7A) or 3-aminobenzaimde (3-AB) (Figure 7B), suggesting a significant role of PARP in this effect of NADPH.

4.4. Altered calcium homeostasis is involved in the effects of NADPH on C6 glioma cell survival

By applying the Ca^{2+} chelator BAPTA-AM, we also obtained evidence suggesting a role of altered calcium homeostasis in the effects of NADPH on C6 glioma cells: The NADPH-induced decreases in C6 glioma cell survival were partially prevented by treatment of the cells with BAPTA-AM (Figure 8).

5. DISCUSSION

The key findings of this study include: First, treatment of NADPH can significantly decrease the survival of glioma cells, while it does not affect the survival of primary astrocyte cultures; second, oxidative stress generated by such enzymes as NADPH oxidase at least partially mediates the effects of NADPH on tumor survival, and PARP activation is one of the downstream effectors of the oxidative stress; and third, altered calcium homeostasis partially contributes to the effects of NADPH on the cell survival.

Our study has indicated that as low as 1 μM NADPH can induce significant decreases in the number of surviving C6 glioma cells, as shown by our LDH assay, Trypan blue assay and flow cytometry-based PI assay. These results have clearly demonstrated that NADPH has the novel property that it can induce decreases in glioma cell survival. Because we have also found that NADPH at the concentration of 1 mM can not decrease the survival of primary astrocyte cultures, NADPH appears to selectively decrease the survival of tumor cells. These results suggest that tumor cells have characteristic properties that underlie the responses of the cells to NADPH.

Our study has indicated that NADPH can significantly increase cell necrosis, as assessed by the flow cytometry-based PI assay. However, this study might

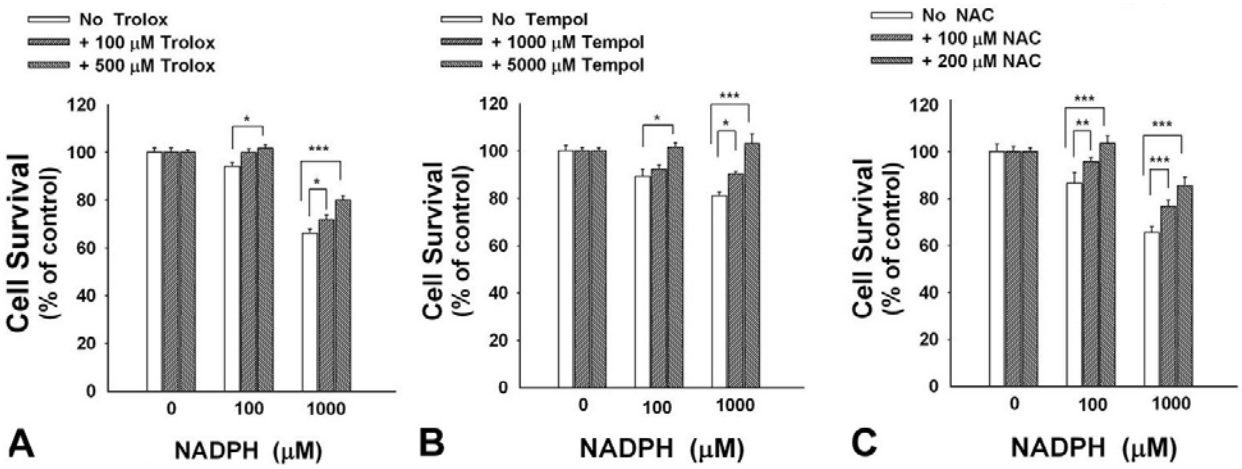

Figure 5. Antioxidants attenuated the effects of NADPH on the survival of C6 glioma cells. The cells were pre-treated with Trolox (A), Tempol (B) or N-acetyl cysteine (C) for 30 min, followed by co-treatment with 0.1 or 1 mM NADPH for 24 hrs. The percentage of cell survival was assessed by LDH assay. N = 11-24. Data were pooled from 3 independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

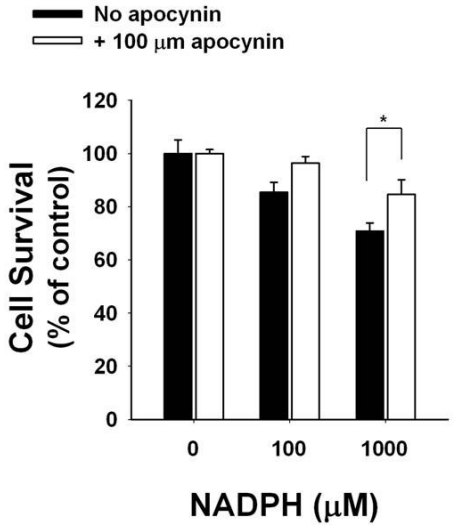

Figure 6. The NADPH oxidase inhibitor apocynin attenuated the effects of NADPH on the survival of C6 glioma cells. The cells were pre-treated with 0.1 mM apocynine for 30 min, followed by co-treatment with 0.1 or 1 mM NADPH for 24 hrs. The percentage of cell survival was assessed by LDH assay. N= 9. Data were pooled from 3 independent experiments. *, $p < 0.05$.

underestimate the extent of cell necrosis in the cultures, since the necrotic cells may be burst even before our PI assay. Our study has further suggested that the NADPH-induced death of glioma cells is mainly necrosis, but not apoptosis: There is no any significant decrease in nuclear size of the NADPH-treated C6 glioma cell cultures. Because nuclear condensation is a hallmarker of cell apoptosis (13), this observation argues against the possibility that NADPH affects glioma cell survival mainly by inducing apoptosis.

Our study has strongly indicated that oxidative stress mediates the effect of NADPH on the cell survival: As shown by the DHE assay, NADPH can induce marked increases in ROS in the cells at both 3 and 24 hours after the treatment; and all of the three structurally different antioxidants --- Trolox, Tempol and N-acetyl cysteine --- can significantly attenuate the effects of NADPH. The nearly complete prevention of the cell death by the treatment of Tempol --- a membrane permeable superoxide scavenger --- suggests that oxidative stress plays a key role in the effect of NADPH on the cell survival. Because oxidative stress is one of the major factors in cell death under a variety of physiological and pathological conditions (14-16), it is not surprising that ROS also plays a significant role in the tumor cell death induced by NADPH.

NADPH oxidase --- an enzyme on the plasma membranes --- is a major enzyme for ROS generation (3-5).

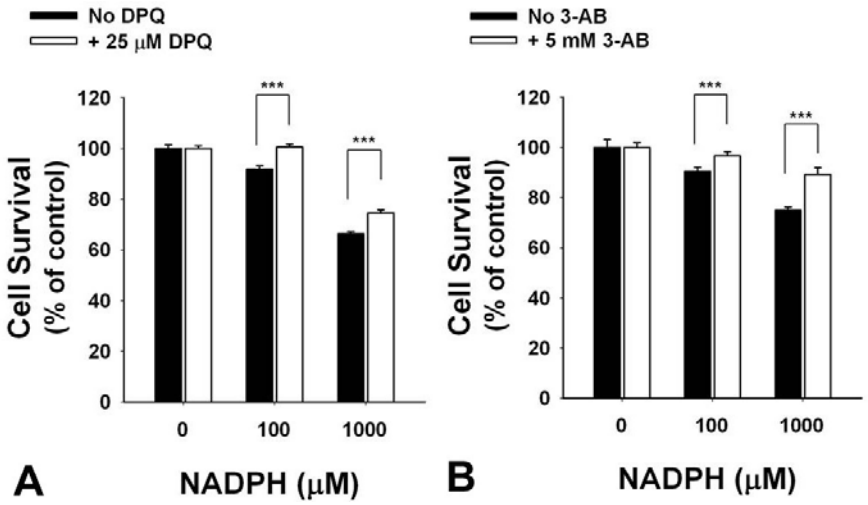

Figure 7. Evidence suggesting a role of PARP in NADPH-induced decreases in C6 glioma cell survival. The cells were pre-treated with DPQ (A) or 3-aminobenzaimde (3-AB) (B) for 30 min, followed by co-treatment with 0.1 or 1 mM NADPH for 24 hrs. N= 20. Data were pooled from 3 independent experiments. ***, $p < 0.001$.

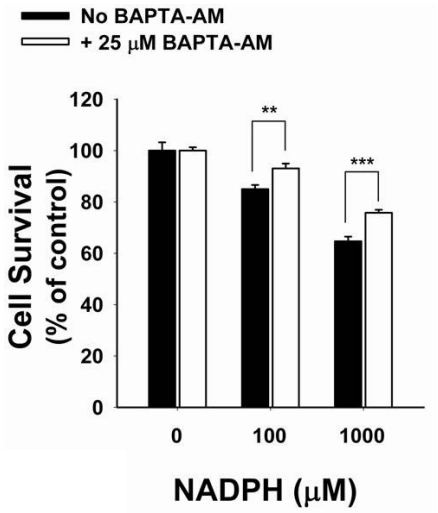

Figure 8. Evidence suggesting a role of altered calcium homeostasis in NADPH-induced decreases in C6 glioma cell survival. The cells were pre-treated with the Ca^{2+} chelator BAPTA-AM for 45 min, followed by co-treatment with 0.1 or 1 mM NADPH for 24 hrs. The percentage of cell survival was assessed by LDH assay. N = 20. Data were pooled from 3 independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

It has been found that the enzyme is not only expressed in immune cells, but also expressed in a wide variety of cell types (3-5). It has been found that NADPH oxidase is expressed in certain types of tumor cells (3-5). Our results have suggested that NADPH oxidase plays a significant role in the effects of NADPH on the glioma cell survival, as indicated by the capacity of apocynin --- a specific inhibitor of NADPH oxidase --- to decrease the effects of NADPH. Many studies have indicated PARP-1 activation as a key factor in cell death induced by oxidative stress both *in vitro* and *in vivo*, which leads to cell death by such mechanisms as depleting intracellular NAD^+, increasing mitochondrial permeability transition (17-19) and inducing nuclear translocation of apoptosis-inducing factors (20). Our current study has suggested that PARP activation contributes to the effects of NADPH on the cell survival, as indicated by the capacity of two PARP inhibitors to attenuate the effects of NADPH. Collectively, our study has suggested the following pathway of NADPH-induced decreases in glioma cell survival: NADPH treatment leads to NADPH oxidase-dependent generation of ROS and subsequent PARP activation, resulting in decreased cell survival.

Because oxidative stress may induce altered calcium homeostasis, which could significantly contribute to the cell death. Indeed, our study using the calcium chelator BAPTA-AM has indicated the capacity of the Ca2+ chelator to decrease the effects of NADPH, suggesting a significant role

of altered calcium homeostasis in the effects of NADPH on the cell survival.

Our study indicates that NADPH decreases selectively the survival of glioma cells, while it does not affect the survival of primary astrocyte cultures. This characteristic property of NADPH suggests that that NADPH may be used to treat gliomas. Malignant glioma is a major type of brain cancer, and most of the patients of malignant glioma have poor prognosis (21, 22). It is of great importance to find new approaches to kill glioma cells. Our study has suggested that NADPH is a novel agent that can profoundly decrease the cell survival of C6 glioma cells, thus suggesting that NADPH might become a new drug that may be used for treating cancer. Future studies are warranted to determine the effects of NADPH on tumor survival in animal models.

6. ACKNOWLEDGMENTS

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