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Involvement of Oxidative Stress and Poly(ADP-ribose) Polymerase Activation in 3-Nitropropionic Acid-induced Cytotoxicity in Human Neuroblastoma Cells

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3-Nitropropionic acid (3-NP) inhibits electron transport in mitochondria, leading to a metabolic failure. In order to elucidate the mechanism underlying this toxicity, we examined a few biochemical changes possibly involved in the process, such as metabolic inhibition, generation of reactive oxygen species (ROS), DNA strand breakage, and activation of Poly(ADP-ribose) polymerase (PARP). Exposure of SK-N-BE(2)C neuroblastoma cells to 3-NP for 48 h caused actual cell death, while inhibition of mitochondrial function was readily observed when exposed for 24 h to low concentrations (0.2~2 mM) of 3-NP. The earliest biochemical change detected with low concentration of 3-NP was an accumulation of ROS (4 h after 3-NP exposure) followed by degradation of DNA. PARP activation by damaged DNA was also detectable, but at a later time. The accumulation of ROS and DNA strand breakage were suppressed by the addition of glutathione or N-acetyl-L-cysteine (NAC), which also partially restored mitochondrial function and cell viability. In addition, inhibition of PARP also reduced the 3-NP-induced DNA strand breakage and cytotoxicity. These results suggest that oxidative stress and activation of PARP are the major factors in 3-NP-induced cytotoxicity, and that the inhibition of these factors may be useful in protecting neuroblastoma cells from 3-NP-induced toxicity.

Key Words: 3-Nitropropionic acid, Cytotoxicity, Oxidative stress, DNA damage, Poly(ADP-ribose) polymerase, 3-Aminobenzamide

INTRODUCTION

3-Nitropropionic acid (3-NP), a plant and fungal toxin, irreversibly inhibits succinate dehydrogenase of the mitochondrial electron transport chain, resulting in a failure of oxidative phosphorylation (Alston et al, 1977) and probably leading to increased neuronal vulnerability to external stimulations. Systemic administration of 3-NP induced acute encephalopathy in the basal ganglia or selective lesion in the striatum (Ludolph et al, 1991; Beal et al, 1993; Borlongan et al, 1997; Sugino et al, 1997), similar to symptoms observed in Huntington's disease. In addition, recent studies showed that 3-NP induced apoptosis in cultured striatal and cortical neurons (Behrens et al, 1995; Ohgoh et al, 2000). Although the underlying mechanisms of the brain damages caused by 3-NP are not well understood, inhibition of mitochondrial energy production (Alston et al, 1977), oxidative stress (Binienda & Kim, 1997), and Ca^{2+} mobilization (Deshpande et al, 1997) have been implicated.

Poly(ADP-ribose) polymerase (PARP), a nuclear enzyme, is activated by free DNA ends generated by a variety of environmental stimuli, such as oxygen free radicals (Szabo et al, 1996a; Szabo et al, 1996b). When activated, the enzyme catalyzes a serial addition of ADP-ribose to cellular proteins and DNA using NAD^+ as a substrate, preventing further deterioration of cellular components. This poly (ADP-ribosyl) ation is suggested to be one of the mechanisms involved in a variety of physiological events such as chromatin condensation, DNA replication, gene expression, malignant transformation, cellular differentiation, and apoptosis (Herceg & Wang, 2001; Tentori et al, 2002). In an over-activated state, however, the enzyme consumes a large amount of NAD^+, resulting in a depletion of ATP and frequently changing the fate of the cells subjected to it (Schraufstatter et al, 1986a; Szabo et al, 1996b). Recently, an enhancement of immunoreactivity to poly (ADP-ribosyl) ation was reported within 3-NP-administered striatum, suggesting an involvement of poly (ADP-ribosyl)ation in 3-NP-induced neuronal damage (Sugino et al, 1997). Nevertheless, the eventual outcome of PARP activation in 3-NP-induced striatal damage has not been thoroughly

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ABBREVIATIONS: 3-NP, 3-nitropropionic acid; PARP, poly(ADP-ribose) polymerase; ROS, reactive oxygen species; DCFH-DA, 2',7'-dichlorofluorescin diacetate; PBN, α-phenyl-N-tert-butylnitrone; NAC, N-acetyl-L-cysteine; 3-AB, 3-aminobenzamide.

explored.

In the present study, we (*i*) found that 3-NP-induced mitochondrial metabolic inhibition and further neuronal cell death were mediated by an accumulation of free radicals which were attenuated by glutathione (GSH) and N-acetyl-L-cysteine (NAC), (*ii*) confirmed the involvement of PARP, and (*iii*) presented possibilities of PARP inhibitors as a useful reagent for rescuing cells from cytotoxicity induced by 3-NP.

METHODS

Chemicals

3-NP, N-acetyl-L-cysteine (NAC), GSH ethyl ester, trolox, α-phenyl-N-tert-butylnitrone (PBN) and 3-aminobenzamide (3-AB) were purchased from Sigma Chemical Company, tissue culture plates and materials were from GIBCO Laboratories, [^{3}H]-NAD$^+$ was from DuPont NEN, and 2', 7'-dichlorofluorescin diacetate (DCFH-DA) and 2',7'-dichlorofluorescein (DCF) were from Eastman Kodak. All drugs were dissolved in phosphate buffered saline (PBS; pH 7.4 at 25°C).

Cell culture

Human neuroblastoma SK-N-BE(2)C cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified chamber of 5% CO_2 at 37°C. A day before each treatment, growth medium was replaced by a serum free medium supplemented with 5 μg/ml each of insulin and transferrin.

Determination of metabolic inhibition

Metabolic inhibition or mitochondrial dysfunction was determined by measuring the ability of cells to reduce 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) bromide. Thus, approximately 5×10^3 cells per well in a 96 well culture plate were treated for the period of time indicated in each experiment. After treatments, the drug-containing medium was replaced with 100 μl of fresh medium containing MTT (0.5 mg/ml) and incubated at 37°C for 2 h. After incubation, 100 μl of DMSO were added to each well, and color was developed at room temperature by gentle shaking for 15 min and absorbancies at 570 nm were measured by ELISA reader.

Determination of cytotoxicity

To quantify cell death, the amount of lactate dehydrogenase (LDH) released into the bathing medium was measured (Bergmeyer & Bernt, 1974). Approximately 1×10^5 cells per well in a 24 well culture plate were treated for the period of time indicated in each experiment. A culture sample was centrifuged at 800 *g* for 5 min, and an aliquot of the supernatant (100 μl) was mixed with 100 μl of LDH assay solution containing 100 mM potassium phosphate (pH 7.5), 0.4 mg/ml sodium pyruvate, and 0.2 mg/ml NAD$^+$. Samples were incubated at room temperature for 20 min and then analyzed spectrophotometrically at 340 nm. Cells were treated with 0.1% Triton X-100 to determine cellular LDH content. and relative amounts of LDH released were calculated as (sample LDH − blank)/(total LDH − blank) × 100(%).

Generation of ROS

Generation of intracellular ROS was measured according to the method developed by Diez-Fernandez et al. (1999) using 2',7'-dichlorofluorescin diacetate (DCFH-DA). After treatment, cells on the culture dishes were washed with Mg^{2+}-free Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2$, 5.6 mM glucose, 5 mM Hepes, pH 7.4), and incubated in 2 ml of same buffer containing DCFH-DA (5 μM) at 37°C for 30 min with agitation to load DCFH-DA into the cells. After loading, cells were washed with same buffer without DCFH-DA to remove residual DCFH-DA and detached immediately with trypsin/EDTA. Fluorescence retained within cells was measured using fluorospectrometer (SFM 25, Kontron, Switzerland) at excitation of 488 nm and emission of 525 nm.

Determination of single strand DNA breaks

To estimate the amount of undamaged, double-stranded DNA, samples were divided into 3 tubes. DNA samples were isolated from variously treated cells, and minimum fluorescence value (F_{min}) was obtained from DNA sonicated for 60 sec before incubation at pH 12.4, and maximum value (F_{max}) was obtained from samples kept at pH 11.0, which is below the pH needed for unwinding without sonication. Subsequently, these pH-adjusted samples were incubated at 0°C for 30 min followed by incubation at 15°C for 15 min. The reaction was terminated by lowering pH down to 11.0 and ethidium bromide (6.7 μg/ml) was added. Fluorescence of each tube was measured by Perkin-Elmer fluorescence spectrophotometer with an excitation wavelength of 520 nm and an emission wavelength of 590 nm. Results are expressed as % double-stranded DNA = $(F-F_{min})/(F_{max}-F_{min})\times100$ (Birnboim & Jevcak, 1981).

PARP activity

Harvested cells were permeabilized for 10 min in 56 mM Hepes (pH 7.5) containing 28 mM KCl, 28 mM NaCl, 2 mM $MgCl_2$, 0.01% digitonin, 125 nmol of NAD$^+$, and 0.25 μCi of [^{3}H]NAD$^+$ (1 Ci ~ 3.7 GBq). Permeabilized cells were incubated for 5 min at 37°C for ribosylation to occur using [^{3}H]NAD$^+$, and proteins were precipitated with 200 μl of 50% (w/v) trichlorolacetic acid (TCA). After two washes with TCA, protein pellets were solubilized in 2% (w/v) SDS in 0.1 M NaOH at 37°C overnight and radioactivity was measured by scintillation counter (Schraufstatter et al, 1986b).

Statistical analysis

Four to five independent experiments were carried out for each condition. All data were analyzed by one-way analysis of variance (ANOVA) with Scheffe's multiple comparison tests to compare means between groups. Significance was taken when $P<0.05$.

RESULTS

Mitochondrial dysfunction and cytotoxicity by 3-NP

Metabolic suppression of mitochondria and cytotoxicity of human neuroblastoma cells, SK-N-BE(2)C, after exposure to low (0.2~2 mM) and high concentrations (10 mM) of 3-NP were examined by MTT and LDH assay, respectively. Exposure of the cells to 0.2 mM to 2 mM of 3-NP for 24 h resulted in dose-dependent metabolic suppression (Fig. 1A). Since 3-NP is a mitochondrial metabolic inhibitor, this result may not necessarily reflect the degree of cytotoxicity. Therefore, In order to determine the actual cytotoxicity of 3-NP, LDH release to the culture medium following 3-NP exposure was determined. The amount of LDH released was not readily noticeable upon 24 h exposure (data not shown). However, LDH release became significantly obvious at 48 h exposure, and were 17.7±3.7% and 52.8±9.6% of the control value upon exposure to 0.8 mM and 10 mM of 3-NP, respectively (Fig. 1B). These results suggest that the mitochondrial metabolic suppression occurs with a low dosage of 3-NP, however, the actual cell death occurs at a later time point.

Involvement of ROS in 3-NP-induced cytotoxicity

Due to its functional nature as succinate dehydrogenase inhibitor and mitochondrial electron transport chain blocker, 3-NP-induced metabolic suppression was expected

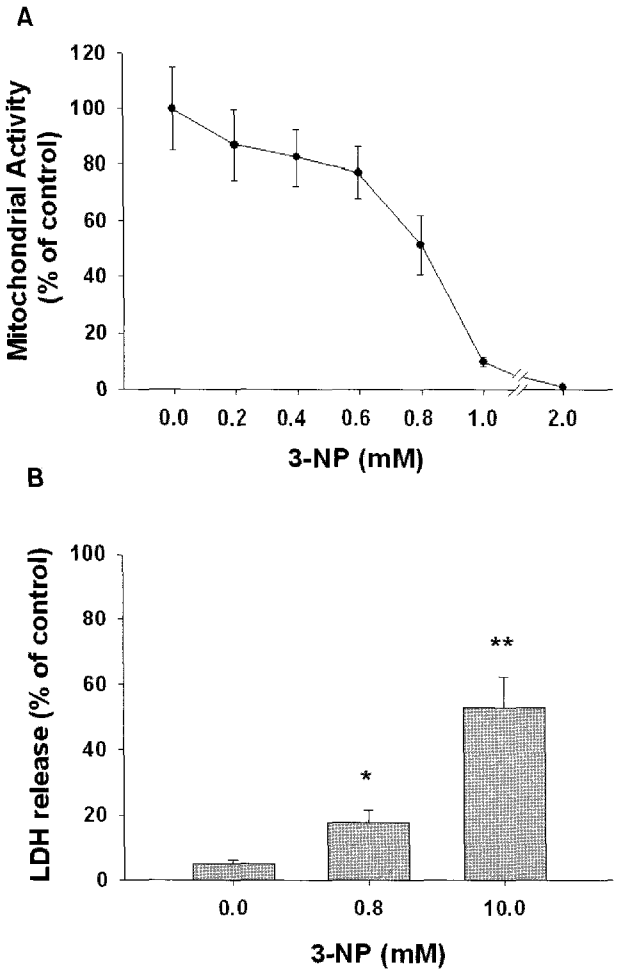

Fig. 1. Mitochondrial activity and cytotoxicity of 3-NP to human neuroblastoma cells. Cells grown in 96-well plates were exposed to 0.2~2 mM of 3-NP for 24 h, and mitochondrial activities were measured using MTT reduction assay as described in the text (A). Cytotoxicities of 0.8 mM and 10 mM 3-NP to neuroblastoma cells were determined by measuring the amount of LDH released in 48 h (B). Values at each concentration are expressed as percentage of control value. Results are mean±SE from five separate experiments of triplicates. *indicates $p<0.05$ and **indicates $p<0.01$ compared with the control.

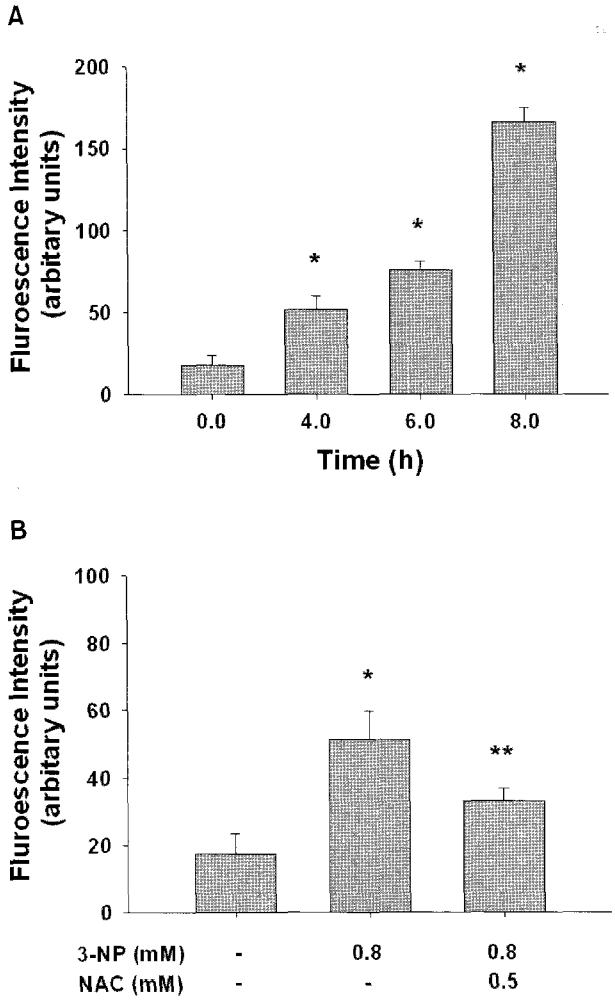

Fig. 2. Effect of 3-NP on ROS generation and inhibition by NAC. After 0.8 mM 3-NP exposure in the presence and absence of 0.5 mM NAC, cells were loaded with DCFH-DA at 37°C for 30 min. After loading, cells were washed to remove residual DCFH-DA and then detached from culture dishes. Intensities of fluorescence generated by oxidation of DCFH were measured at different time points after 3-NP exposure (A) and 4 h later in the presence or absence of antioxidant NAC (B). Results are mean±SE from five separate experiments. *indicates $p<0.05$ compared with the control value and **indicates $p<0.05$ compared with the values obtained from exposure of 3-NP alone.

to result in an accumulation of ROS. To test this hypothesis, cell permeable dye DCFH-DA, which becomes fluorescent upon oxidation, was loaded into the cells after 3-NP insult and the change of fluorescence was measured by fluorometer at specified times. As shown in Fig. 2A, elevation of fluorescence became obvious as early as 4 h after the exposure to 3-NP, and was approximately 8-fold higher at 8 h after stimulation. The 3-NP-induced DCF fluorescence, however, was significantly reduced by treatment of NAC when measured 4 h after 3-NP exposure (Fig. 2B).

Protection of 3-NP-induced metabolic inhibition and cytotoxicity by antioxidants

In order to elucidate a role of ROS thus generated, antioxidants, such as GSH and NAC were tested for their ability to reverse mitochondrial metabolic suppression and to rescue cells from the toxicity. Thus, cells treated with antioxidants 1 h prior to low concentration of 3-NP exposure exhibited significant recoveries, showing metabolic activities of 80.5±11.7% and 72.2±1.6% with NAC and GSH, respectively, while 3-NP without antioxidant resulted in an MTT reduction of 45.2±0.4% (Fig. 3A). Pre-treatment of the cells with antioxidants also reversed the cytotoxicity induced by subsequent exposure to a high concentration of 3-NP (10 mM), as determined by the amount of LDH released (Fig. 3B). These results suggest

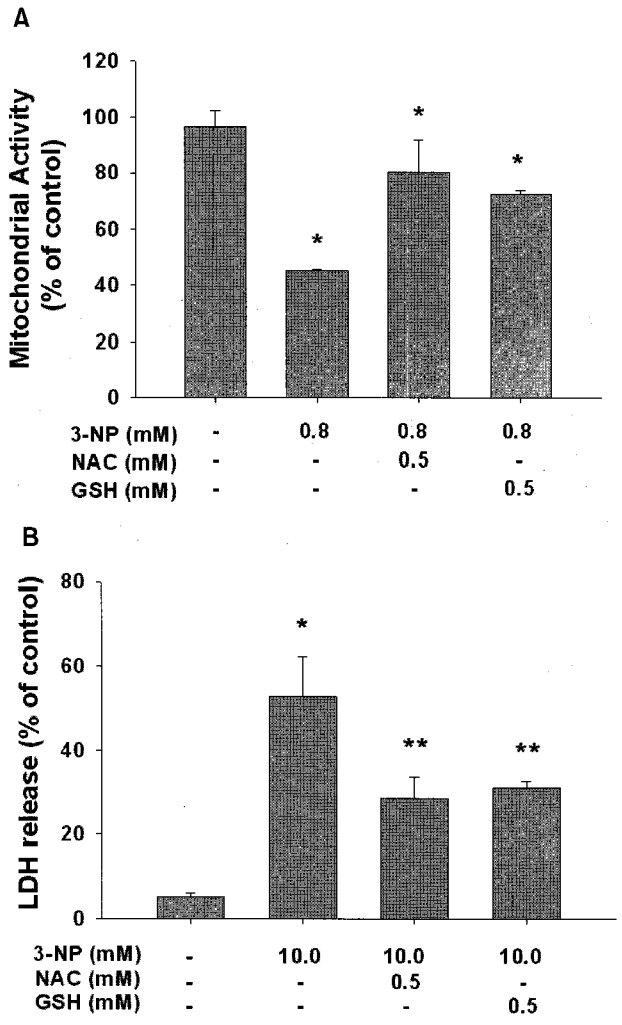

Fig. 3. Effects of antioxidants on 3-NP-induced mitochondrial activity and cytotoxicity. Cells pretreated with GSH ethyl ester or NAC for 1 h were exposed to 3-NP (0.8 mM) for 24 h, and mitochondrial activities were determined by MTT assay (A). Viabilities of similarly treated cells exposed to 3-NP for 48 h were measured by LDH assay, and values are expressed as percentage of those of untreated cells (B). Results are mean±SE from five separate experiments of triplicates. In both panels, *indicates $p<0.05$ compared with the control value and **indicates $p<0.05$ compared with the values obtained from exposure of 3-NP alone.

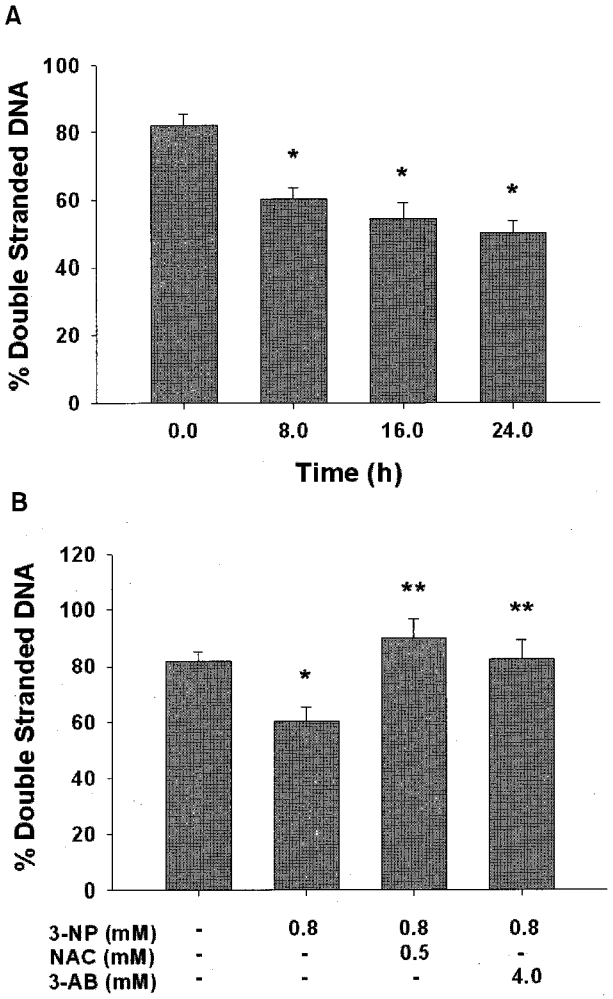

Fig. 4. Effects of 3-NP on DNA strand breakage, and inhibition by NAC and PARP inhibitor 3-AB. Cellular DNA was isolated from variously treated SK-N-BE(2)C cells and single-strand DNA breaks were determined by the fluorescence analysis of alkaline-induced unwinding of DNA, as described in the text. Amount of double-stranded undamaged DNA in cells exposed to 0.8 mM 3-NP decreased time-dependently (A). DNA strand breakage induced by exposure to 3-NP for 8 h was efficiently inhibited by pretreatment of NAC or 3-AB (B). Data are mean±SE from five independent experiments of duplicates. *indicates $p<0.05$ compared with the value of 0 h time point and control in panels A and B, respectively and **in panel B indicates $p<0.05$ compared with the value obtained from exposure of 3-NP alone.

that the metabolic inhibition and cell death induced by 3-NP were mediated partially by the ROS generated.

Effect of 3-NP on DNA strand breakage

Since majority of macromolecules within cells can be targets of inappropriately produced ROS, we assumed that cellular DNA might have also been cleaved upon exposure to 3-NP. Therefore, levels of single-strand DNA breaks in 3-NP treated cells were determined by fluorescence analysis of alkaline-mediated DNA. As shown in Fig. 4A, cells exposed to 0.8 mM 3-NP for 8 h showed 60.3±3.4% of double-stranded DNA, while most of the DNA in the control cells were undamaged (<18%). Amount of undamaged DNA was slightly but gradually reduced during 16 h after 3-NP exposure, suggesting that an accumulation of ROS responsible for DNA strand breakage occurred rather earlier. However, treatment with either NAC or GSH from 1 h prior to 3-NP exposure significantly reduced the amount of single-strand DNA breaks and increased the quantity of undamaged DNA in 3-NP-exposed cells (Fig. 4B), suggesting that ROS generated by 3-NP was responsible for DNA damage. Furthermore, to our surprise, pretreatment of the cells with PARP inhibitor 3-AB also suppressed the amount of single-strand DNA breaks down to a basal level.

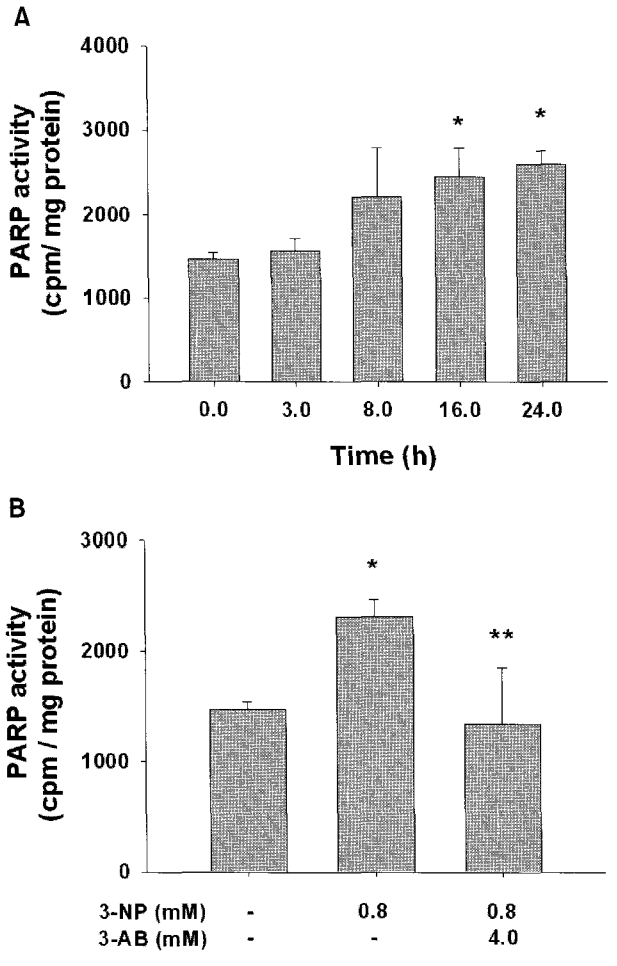

Fig. 5. PARP activation by 3-NP and inhibition by 3-AB. SK-N-BE(2)C cells permeabilized with 0.01% digitonin were incubated in the presence of [^{3}H]NAD$^+$ for ribosylation to occur. After wash and precipitation of protein, amount of radioactivity incorporated into cellular protein by activated PARP was measured, as described in the text. Amount of radioactivity incorporated into cellular protein at different time points were measured as an index of PARP activity in SK-N-BE (2)C cells exposed to 0.8 mM 3-NP (A). Digitonin permeabilized cells were exposed to 3-NP alone or 3-NP with 4 mM 3-AB, and the radioactivity incorporated into cellular proteins was measured 24 h later (B). Data are mean±SE from four different experiments of duplicates. *indicates $p<0.05$ compared with the value of 0 hr time point in panel A and control in panel B respectively, and **in panel B indicates $p<0.05$ compared with the value obtained from exposure of 3-NP alone.

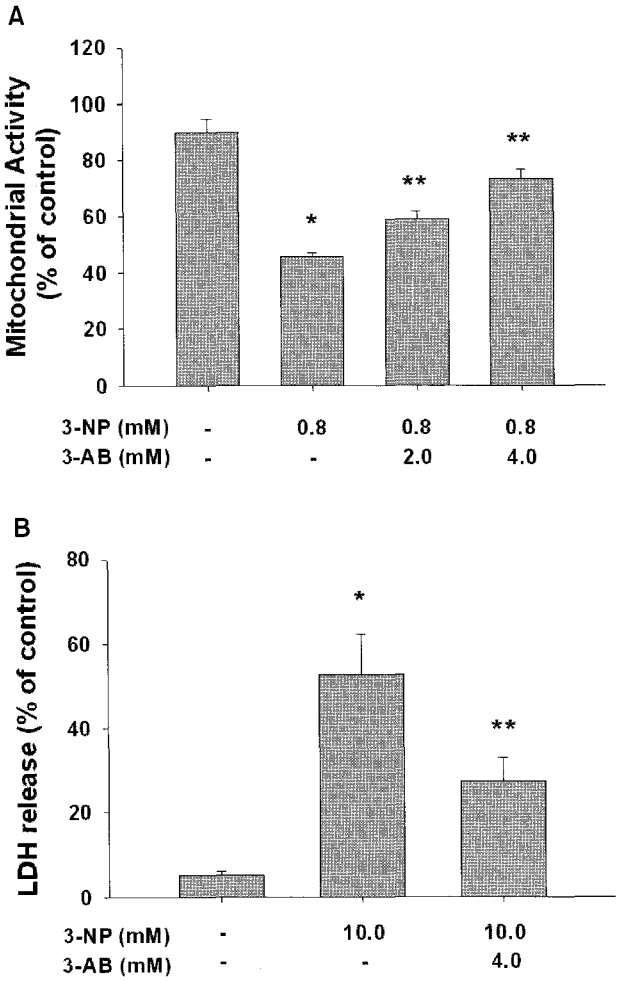

Fig. 6. Effect of PARP inhibition on 3-NP-induced mitochondrial activity and cytotoxicity. SK-N-BE(2)C cells were pre-exposed to 2 or 4 mM 3-AB 1 hr prior to exposure to 3-NP for 24 h. Mitochondrial activities were determined using the MTT assay, and is expressed as a percentage of untreated cells (A). Attenuation of cell death by inhibition of PARP was determined by measuring the amount of LDH released into the bathing medium after 3-NP treatment with or without 4 mM 3-AB for 48 h (B). Data are mean±SE from four different experiments of triplicates. In both panels *indicates $p<0.05$ compared with values obtained from untreated cells and **indicates $p<0.05$ compared with values from exposure of 3-NP alone.

Involvement of PARP in 3-NP induced cytotoxicity

Fragmentation of DNA by 3-NP exposure suggests that PARP activation is a very likely candidate for the mediator of 3-NP-induced toxicity. To test this hypothesis, we determined time course of PARP activity by measuring poly (ADP-ribosyl)ation of cellular proteins, that is, incorporation of ^{3}H from added [^{3}H]NAD^{+} into cellular proteins. Elevation of radioactivity incorporation was noticeable as early as 8 h and became significant at 16 h after 3-NP exposure (Fig. 5A), but was suppressed by treatment of PARP inhibitor 3-AB (Fig. 5B). Inhibition of PARP activity by 3-AB partially restored cellular metabolism in a dose-dependent manner as determined by the amount of MTT reduced (Fig. 6A) as well as rescuing cells from death (Fig. 6B).

DISCUSSION

Cell death caused by an exposure to 3-NP is due to the suppression of electron transport within mitochondria (Ohgoh et al, 2000), although biochemical changes involved in the cytotoxicity have not yet been completely defined. In this study, metabolic inhibition or mitochondrial dysfunction was determined by the amount of MTT reduced which was proportional to the amount of 3-NP added. These measurements, however, seem to overestimate the toxicity, since the metabolic inhibition and/or mitochondrial dysfunction reached to as high as 100% with 1 mM 3-NP in 24 h, but the amount of LDH released was barely detected in this experimental paradigm. These results suggest that although metabolic inhibition with sub-lethal concentrations of 3-NP occurs quickly after administration, initiation of the cell death program and disintegration of the membrane occur at a much later time point. For investigation of cellular responses to 3-NP in detail, mitochondrial dysfunctions were examined using different concentrations of 3-NP. At lower concentrations of 3-NP (0.2~2 mM), the metabolic activities, as determined by degree of MTT reduction, were decreased in a dose dependent manner. A sudden decrease of the amount of MTT reduction upon exposure to 0.6 mM to 1 mM 3-NP suggests a buffering threshold of the cells at these concentrations. In addition, the accumulation of ROS was detected 4 h after 3-NP exposure. Therefore, the cells must have enough endogenous antioxidants to detoxify ROS accumulated within 4 h by up to 0.6 mM 3-NP. The buffering capacity of SK-N-BE(2)C cells was further evidenced by the delayed onset of PARP activation. The onset of PARP activation by 3-NP was slow compared with other studies in which oxygen radicals, such as hydroxyl radicals, hydrogen peroxide or peroxinitrite were directly added to culture medium (Radons et al, 1994; Coppola et al, 1995; S zabo et al, 1996a; Szabo et al, 1996b). In those studies, DNA damage and PARP activation were obvious within 1~2 h after oxyradical treatment. However, in our experimental conditions, it took at least 8 h for DNA cleavage to occur and 16 h for PARP activation. Assuming that the ROS generated upon 3-NP exposure can initially be removed by endogenous antioxidant systems, it would be plausible to speculate that the activation of cell death program must occur at a much later time point than previously reported. Reduction of 3-NP-induced single strand DNA breakage down to control levels by antioxidant NAC implies that the DNA breakage was one of the fatal outcomes brought about by the accumulated ROS. This speculation was further supported by reversal of MTT reduction and suppression of LDH release by NAC and GSH. The accumulation of ROS was suppressed by exogenously added NAC possibly through sparing the reduction of intracellular GSH level. It was also reported that 3-NP exposure rapidly depleted cellular GSH in cultured neurons (Olsen et al, 1999) and in rat hippocampus (Binienda et al, 1998). Thus, the endogenous GSH level or GSH/GSSG status is likely the prime defensive mechanism against 3-NP-induced toxicity. An intriguing observation in this study, however, was that the antioxidants such as trolox and PBN were neither able to relieve the cells from metabolic inhibition nor to rescue them from 3-NP-induced toxicity. It is not clear presently why these antioxidants were not able to rescue the cells or inhibit LDH release. A recent report by Olsen et al. (1999) showed that 3-NP-induced toxicity was attenuated by pretreatment with 10 mM PBN. But, we were not able to test any further, since PBN concentration higher than 1 mM exhibited cellular toxicity in our system (data not shown).

Suppression of DNA cleavage by PARP inhibitor 3-AB was unexpected, since free DNA ends are shown to be prerequisite for the activation of PARP (Nosseri et al, 1994). A possible explanation for this would be that 3-AB can attenuate the GSH depletion as reported by Mizumoto et al. (1993): that is, 3-AB restored cellular GSH levels and prevented the cells from death in methyl methansulfonate-treated hepatocytes. However, more direct evidence is needed concerning the role of 3-AB in intracellular GSH metabolism in 3-NP-exposed cells. In contrast, the ability of 3-AB to rescue cells from death in ischemia-reperfusion injuries where xanthine oxidase as the source of ROS, was reported (Aalto et al, 1993). In the above study, adenine nucleotide was depleted from umbilical vein endothelial cells upon exposure to xanthine oxidase or hypoxanthine, and the depletion was partially prevented by GSH or 3-AB, but not by other hydroxyl radical scavengers. These results are similar to ours in that no other antioxidants, but NAC/GSH or 3-AB, were able to reverse the toxicity. This consistency raises another possibility that 3-AB may be able to act as a safeguard, monitoring an over-activation of cellular metabolism. A large amount of ATP is required for maintaining high levels of intracellular GSH. However, fragmentation of DNA results in an over-activation of PARP which will consume a large amount of ATP. Therefore, the inhibition of PARP activity preserved the intracellular GSH levels (Marini et al, 1993), most likely due to a less consumption of cellular NAD and ATP. Partial rescue from cell death by 3-AB treatment, as determined by the amount of LDH released, further supports this hypothesis.

The results described herein may not be sufficient to conclude that protective mechanism of 3-AB in 3-NP-induced cytotoxicity is closely related to PARP inhibition. Inhibition of PARP by 3-AB under a controlled condition is generally accepted (Banasik et al, 1992), however, other activity such as cytoprotection has been described in culture when millimolar concentrations of 3-AB were used. For example, some PARP inhibitors, including 3-AB have been shown to affect cell viability, glucose metabolism and DNA synthesis (Milam & Cleaver, 1984), and to inhibit protein kinase C (Ricciarelli et al, 1998), macrophage activation and inducible nitric oxide synthase (Le Page et al, 1997; Le Page et al, 1998). Also, it was reported that 3-AB inhibits the cytochrome P450-dependent metabolism of

chemicals rather than inhibition of PARP (Eriksson et al, 1996). Further studies using more specific PARP inhibitors are needed to clearly demonstrate the involvement of PARP activation in 3-NP-induced cytotoxicity.

Taken together, we have shown that the oxidative stress, DNA strand break, and PARP activation were involved in sequence in sub-lethal doses of 3-NP-induced cytotoxicity. Additionally, antioxidants GSH and NAC, and PARP inhibitor 3-AB effectively protected human neuroblastoma cells from 3-NP-induced toxicity.

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REFERENCES

Aalto TK, Raivio KO. Nucleotide depletion due to reactive oxygen metabolites in endothelial cells: effects of antioxidants and 3-aminobenzamide. *Pediatr Res* 34: 572−576, 1993

Alston TA, Mela L, Bright HJ. 3-Nitropropionate, the toxic substance of Indigofera, is a suicide inactivator of succinate dehydrogenase. *Proc Natl Acad Sci USA* 74: 3767−3771, 1977

Banasik M, Komura H, Shimoyama M, Ueda K. Specific inhibitors of Poly(ADP-ribose) synthetase and mono (ADP-ribosyl) transferase. *Biol Chem* 267: 1569−1575, 1992

Beal MF, Brouillet E, Jenkins BG, Ferrante RJ, Kowall NW, Miller JM, Storey E, Srivastava R, Rosen BR, Hyman BT. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J Neurosci* 13: 4181−4192, 1993

Behrens MI, Koh J, Canzoniero LM, Sensi SL, Csernansky CA, Choi DW. 3-Nitropropionic acid induces apoptosis in cultured striatal and cortical neurons. *Neuroreport* 15: 545−548, 1995

Bergmeyer HU, Bernt E. Lactate dehydrogenase UV assay with pyruvate and NADH, in: Bergmeyer, H.U. (Eds.), Methods of enzymatic analysis. Vol. 2. Academic Press, New York, pp. 574−579, 1995

Binienda Z, Kim CS. Increase in levels of total free fatty acids in rat brain regions following 3-nitropropionic acid administration. *Neurosci Lett* 230: 199−201, 1997

Binienda Z, Simmons C, Hussain S, Slikker W Jr., Ali SF. Effect of acute exposure to 3-nitropropionic acid on activities of endogenous antioxidants in the rat brain. *Neurosci Lett* 251: 173−176, 1998

Birnboim HC, Jevcak JJ. Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. *Cancer Res* 41: 1889−1892, 1981

Borlongan CV, Nishino H, Sanberg PR. Systemic, but not intraparenchymal, administration of 3-nitropropionic acid mimics the neuropathology of Huntington's disease: a speculative explanation. *Neurosci Res* 28: 185−189, 1997

Coppola S, Nosseri C, Maresca V, Ghibelli L. Different basal NAD levels determine opposite effects of poly (ADP-ribosyl) polymerase inhibitors on H_2O_2-induced apoptosis. *Exp Cell Res* 221: 462−469, 1995

Deshpande SB, Fukuda A, Nishino H. 3-Nitropropionic acid increases the intracellular Ca^{2+} in cultured astrocytes by reverse operation of the Na^{+}-Ca^{2+} exchanger. *Exp Neurol* 145: 38−45, 1997

Diez-Fernandez C, Zaragoza A, Alvarez AM, Cascales M. Cocaine cytotoxicity in hepatocyte cultures from phenobarbital-induced rats: involvement of reactive oxygen species and expression of antioxidant defense systems. *Biochem Pharmacol* 58: 797−805, 1999

Erikson C, Busk L, Brittebo B. 3-Aminobenzamide: Effects on cytochrome P450-dependent metabolism of chemicals and on the toxicity of dichlobenil in the olfactory mucosa. *Toxicol Appl Pharmacol* 136: 324−331, 1996

Herceg Z, Wang ZQ. Functions of Poly(ADP-ribose) polymerase (PARP) in DNA repair, genomic integrity and cell death. *Mutat Res* 477: 97−110, 2001

Le Page C, Pellat-Deceunynck C, Drapier JC, Wietzerbin J. Effects of inhibitors of ADP-ribosylation on macrophage activation. *Adv Exp Med Biol* 419: 203−207, 1997

Le Page C, Sanceau J, Drapier JC, Wietzerbin J. Inhibitors of ADP-ribosylation impair inducible nitric oxide synthase gene transcription through inhibition of NF-B activation. *Biochem Biophys Res Commun* 243: 451−457, 1998

Ludolph AC, He F, Spencer PS, Hammerstad J, Sabri M. 3-Nitropropionic acid-exogenous animal neurotoxin and possible human striatal toxin. *Can J Neurol Sci* 18: 492−498, 1991

Marini M, Frabetti F, Brunelli MA, Raggi MA. Inhibition of Poly (ADP-ribose) polymerization preserves the glutathione pool and reverses cytotoxicity in hydrogen peroxide-treated lymphocytes. *Biochem Pharmacol* 46: 2139−2144, 1993

Milam MK, Cleaver EJ. Inhibitors of poly (adenosine diphosphate-ribose) synthesis: effect on other metabolic processes. *Science* 223: 589−591, 1984

Mizumoto K, Glascott PA Jr., Farber JL. Roles for oxidative stress and poly(ADP-ribosyl)ation in the killing of cultured hepatocytes by methyl methanesulfonate. *Biochem Pharmacol* 46: 1811−1818, 1993

Nosseri C, Coppola S, Ghibelli L. Possible involvement of poly (ADP-ribosyl) polymerase in triggering stress-induced apoptosis. *Exp Cell Res* 212: 367−373, 1994

Ohgoh M, Shimizu H, Ogura H, Nishizawa Y. Astroglial trophic support and neuronal cell death: influence of cellular energy level on type of cell death induced by mitochondrial toxin in cultured rat cortical neurons. *J Neurochem* 75: 925−933, 2000

Olsen C, Rustad A, Fonnum F, Paulsen RE, Hassel B. 3-Nitropropionic acid: an astrocyte-sparing neurotoxin *in vitro*. *Brain Res* 850: 144−149, 1999

Radons J, Heller B, Burkle A, Hartmann B, Rodriguez ML, Kroncke KD, Burkart V, Kolb H. Nitric oxide toxicity in islet cells involves Poly(ADP-ribose) polymerase activation and concomitant NAD+ depletion, *Biochem Biophys Res Commun* 199: 1270−1277, 1994

Ricciarelli R, Palomba L, Cantoni O, Azzi A. 3-Aminobenzamide inhibition of protein kinase C at a cellular level. *FEBS Lett* 431: 465−467, 1998

Schraufstatter IU, Hinshaw DB, Hyslop PA, Spragg RG. Cochrane CG. Oxidant injury of cells. DNA strand-breaks activate polyadenosine diphosphate-ribose polymerase and lead to depletion of nicotinamide adenine dinucleotide. *J Clin Invest* 77: 1312−1320, 1986a

Schraufstatter IU, Hyslop PA, Hinshaw DB, Spragg RG, Sklar LA, Cochrane CG. Hydrogen peroxide-induced injury of cells and its prevention by inhibitors of Poly(ADP-ribose) polymerase. *Proc Natl Acad Sci USA* 83: 4908−4912, 1986b

Sugino T, Nozaki K, Kokime T, Hashimoto N, Kikuchi H. 3-Nitropropionic acid induces poly(ADP-ribosyl)ation and apoptosis related gene expression in the striatum in vivo. *Neurosci Lett* 237: 121−124, 1997

Szabo C, Wong HR, Salzman AL. Pre-exposure to heat shock inhibits peroxynitrite-induced activation of poly (ADP-ribosyl) transferase and protects against peroxynitrite cytotoxicity in J774 macrophages. *Eur J Pharmacol* 315: 221−226, 1996a

Szabo C, Zingarelli B, O'Connor M, Salzman AL. DNA strand breakage, activation of Poly(ADP-ribose) synthetase, and cellular energy depletion are involved in the cytotoxicity of macrophages and smooth muscle cells exposed to peroxynitrite. *Proc Natl Acad Sci USA* 93: 1753−1758, 1996b

Tentori L, Portarena I, Graniani G. Potential clinical applications of Poly(ADP-ribose) polymerase (PARP) inhibitors. *Pharmacol Res* 45: 73−85, 2002