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3-Nitropropionic acid induces poly(ADP-ribosyl)ation and apoptosis related gene expression in the striatum in vivo

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Abstract

Impaired energy metabolism plays an important role in neuronal cell death after brain ischemia, and apoptosis has been implicated in cell death induced by metabolic impairment. In the present study, metabolic impairment was induced by 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase. In order to clarify the involvement of poly(-ADP-ribosyl)ation and apoptotic pathway in 3-NP induced cell death, we examined poly(ADP-ribosyl)ation and the apoptosis related gene protein expression after systemic administration of 3-NP by immunohistochemistry. Poly(ADP-ribosyl)ation was evidently detected in the striatal lesion but not in any other region. Immunoreactive ratio of Bcl-2 to Bax significantly increased both in the striatum and cortex. The data suggest that striatal cell death involves poly(ADP-ribosyl)ation and also apoptotic pathway in part following administration of 3-NP. © 1997 Elsevier Science Ireland Ltd.

Keywords: 3-Nitropropionic acid; Neuronal death; Striatum; Apoptosis; Poly(ADP-ribosyl)ation; Bax; Bcl-2

Two distinct modes of cell death, apoptosis and necrosis, can be distinguished based on differences in morphological, biochemical and molecular changes of dying cells. Necrosis is characterized by a rapid cell swelling and cell lysis, with random degradation of DNA. In contrast, apoptosis or programmed cell death is characterized by cell body shrinkage, cytoplasmic and nuclear fragmentation, and internucleosomal chromatin cleavage [16]. The critical step in the regulated apoptotic DNA fragmentation is the proteolytic inactivation of poly(ADP-ribose) polymerase (PARP) by a group of cysteine proteases with some structural homologies to interleukin-1 beta-converting enzyme. PARP catalyzes the ADP-ribosylation of nuclear proteins at the sites of spontaneous DNA strand breaks and thereby facilitates the repair of this DNA damage. However, the excessive activation of PARP is thought to cause depletion of adenosine 5′-triphosphate and the energy failure resulting in cell death [4,8,19,20].

During the past year the Bcl-2 proto-oncogene has emerged as common regulator of multiple apoptotic pathways [13]. The active form of the Bcl-2 protein, which promotes cell survival, is part of a heterodimer with Bax, which promotes cell death, and the ratio of Bcl-2 to Bax appears to determine the susceptibility to apoptotic stimuli [11,17]. Bcl-2 and Bax proteins are expressed in neurons of the central and peripheral nervous system [7,9].

Systemic administration of 3-nitropropionic acid (3-NP), an irreversible inhibitor of succinate dehydrogenase (complex II of mitochondrial respiratory chain), produces selective basal ganglia lesions [1,2,5,15]. Recent studies indicate that 3-NP may act to induce neuronal apoptosis in vitro and also in vivo [3,12,14], but apoptosis related gene expression has not been reported. The purpose of this study is to examine poly(ADP-ribosyl)ation and the apoptosis related gene protein expression immunohistochemically after systemic administration of 3-NP.

Twenty-four male Sprague–Dawley rats (Shimizu Laboratory supplies, Kyoto, Japan) weighing 280–320 g were used. They were kept in a temperature and light-dark cycle controlled animal room. Lab chow and water were given ad libitum.

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3-NP (Aldrich Chemical, Milwaukee, WI, USA) was dissolved in distilled water to a concentration of 10 mg/ml and pH adjusted to 7.4 with NaOH and administered intraperitoneally 10 mg/kg every 12 h. With this dosing regimen, the animals become acutely ill after the third or fourth injection and show bilateral large striatal lesions, and no rats survived when they were administered more than 50 mg/kg at total doses in our preliminary data. Control animals were given distilled water in the same way.

Since there was variability in the times at which animals became ill, they were clinically examined 2–3 h after the last injections (total doses 30 or 40 mg/kg) and sacrificed after observing their recumbency (group 3, $n = 8$). Other rats were sacrificed 12 h after the second injection (total doses; 20 mg/kg) when they became motionless but there was no obvious behavioral disturbance (group 2, $n = 8$). Control animals were sacrificed on the third day with no symptom (group 1, $n = 8$). The rats were anesthetized with diethyl ether and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). The brains were rapidly removed and cryoprotected in 25% sucrose in PBS overnight at 4°C. Frozen coronal sections (40 μm in thickness) of the brains were prepared. For immunohistochemistry, antibodies against poly(ADP-ribose) (Trevigen, London, UK), Bcl-2, Bax (Santa Cruz Biotechnology, CA, USA) were used. After quenching endogenous peroxidase in 2% H_2O_2 in 60% methanol and blocking with 5% goat serum, sections were incubated overnight at 4°C with polyclonal antibodies against poly(ADP-ribose), Bcl-2, and Bax. The sections were washed in PBS, incubated in biotinylated anti-guinea pig IgG for poly(ADP-ribose) and anti-rabbit IgG antibody for Bcl-2 and Bax at 1:200 dilution for 2 h and in avidin-biotin complex (ABC kit from Vector) for 60 min. Peroxidase was demonstrated with DAB substrate kit (Vector). Negative control sections received identical treatment except for the primary antibody. For evaluation of morphological change, adjacent sections were stained with cresyl violet.

On Nissl-stained sections, large bilateral lesions were detected as pale staining in the striatum in group 3 (Fig. 1). In groups 1 and 2, no striatal lesion was observed.

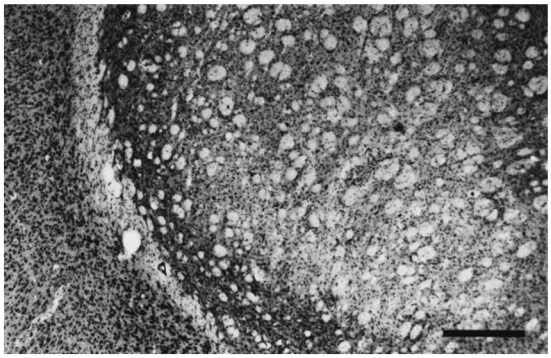

Fig. 1. A photomicrograph of Nissl staining in the left striatal neurotoxic lesion as a consequence of the intraperitoneal administration of the succinate dehydrogenase inhibitor, 3-NP. Scale bar, 1 mm.

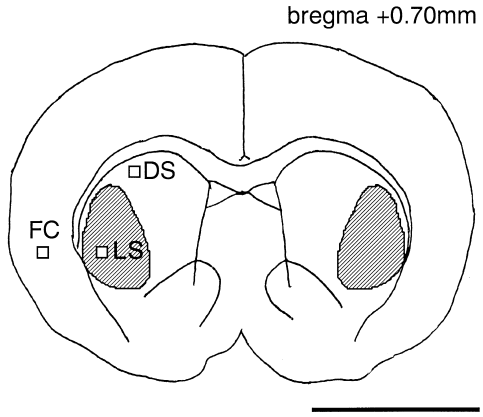

Fig. 2. Location of the striatal lesion. The diagram shows typical extent of striatal lesion following intraperitoneal administration of 3-NP (shaded areas). Cell count was done in the squares indicated in the diagram. LS, lateral striatum; DS, dorsocentral striatum; FC, overlying frontal cortex. Scale bar, 5 mm.

Immunohistochemical changes were examined in coronal brain sections containing striatal lesions. Poly(ADP-ribose)-immunoreactive (IR) cells, Bcl-2-IR cells, Bax-IR cells were counted in serial sections in a blind fashion by independent observers, using the light microscope equipped with a 10× objective. Three rectangular fields (each ca. 1 mm^2) were chosen in lateral striatum, dorsocentral striatum, and frontal cortex (see Fig. 2). Appropriate detection thresholds were then set to provide each counts of poly(ADP-ribose)-IR cells, Bcl-2-IR cells, and Bax-IR cells within these fields by independent observers. The data of the cell count were statistically analyzed by repeated-measures analysis of variance followed by post-hoc Bonferroni test between groups using Stat View II. Data are presented as the mean ± SEM, and when $P < 0.05$, differences were considered significant (Fig. 4).

In groups 1 and 2, no poly(ADP-ribose)-IR was detected (Fig. 3A,B). After the third or fourth injection of 3-NP, poly(ADP-ribose)-IR cells increased significantly in the lateral striatum ($P < 0.01$; Figs. 3C and 4A), and few in the dorsocentral striatum (Fig. 3D). No changes were seen in the number of poly(ADP-ribose)-IR cells in the cortex.

Bcl-2-IR cells were observed moderately in the cortex, and few in the lateral and dorsocentral striatum in control rats (Fig. 3E,F). In groups 2 and 3, Bcl-2-IR cells had a tendency to increase in all three fields we selected with the increase of the doses of 3-NP (Fig. 3G,H). Bcl-2-IR cells in group 3 were significantly more than those in group 1 in the dorsocentral striatum and frontal cortex (Fig. 4B).

Bax immunoreactivity, in turn, was diffusely and abundantly detected both in the striatum and cortex in control animals (Fig. 3I,J). In group 2, no significant change was detected in all three fields we selected. In group 3, Bax-IR cells decreased in the three fields, particularly in the lateral striatal lesion and the dorsocentral striatum compared with group 1 (Figs. 3K,L and 4C). To define whether the immu-

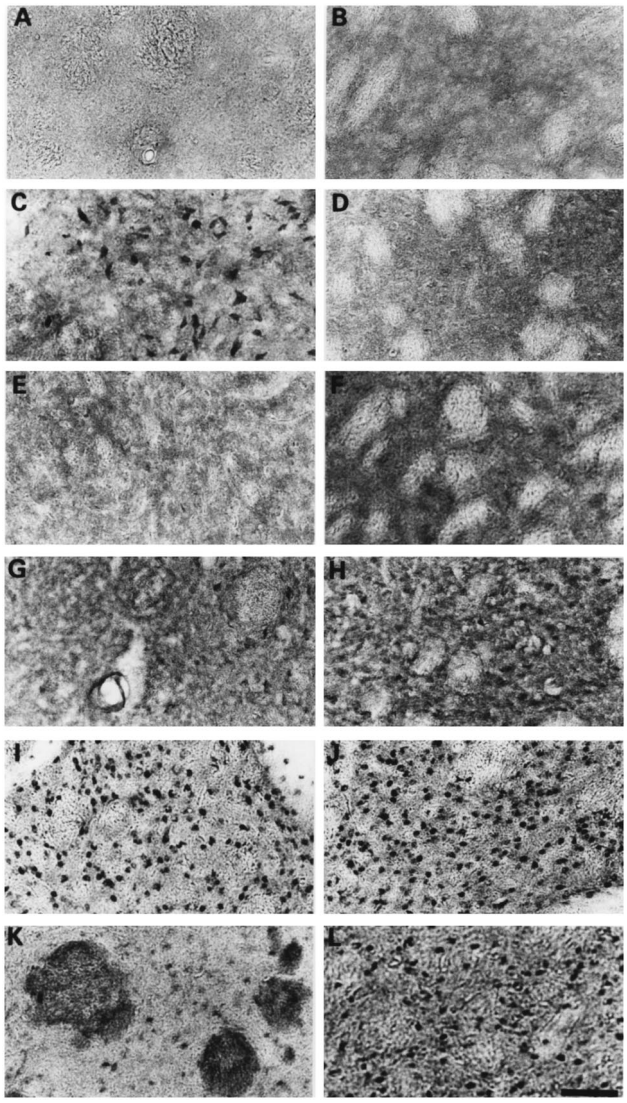

Fig. 3. Photomicrographs of the immunostaining of poly(ADP-ribose) (A–D), Bcl-2 (E–H), and Bax (I–L) in groups 1 (A,B,E,F,I,J) and 3 (C,D,G,H,K,L). Left column shows the photomicrographs in the lateral striatum. Right column shows the photomicrographs in the dorsocentral striatum. Scale bar, 100 μm.

nopositive cells were neurons or not, immunohistochemical study using polyclonal antibody against neuron specific enolase (NSE; Chemicon International, CA, USA) was performed. In this study, adjacent frozen coronal sections (20 μm in thickness) were prepared. Immunostaining was performed as described above. We defined the poly(ADP-ribose)-IR cells as neurons because the immunoreactivity of NSE was detected in the same cells (Fig. 5B,C). Furthermore the configuration of the poly(ADP-ribose)-IR cells in Fig. 5A were identical to neurons. Bcl-2-IR cells, Bax-IR cells were also defined as neurons in the same way (data not shown).

To investigate the role of the expression of apoptosis related gene proteins, Bcl-2 and Bax, Bcl-2–Bax interaction should be considered [11]. As for the ratio of Bcl-2 to Bax, we calculated it by dividing the number of Bcl-2-IR cells by that of Bax-IR cells in each region. It increased in the frontal cortex after the second injection of 3-NP, but was not statistically significant. After the third or fourth administration, the significant increase of the ratio was detected in the lateral and dorsocentral striatum ($P < 0.01$), and frontal cortex ($P < 0.05$).

PARP is activated by DNA breakage, resulting in the addition of up to 100 ADP-ribose groups to substrates, such as histone and PARP itself. Following limited damage of DNA, PARP activation, poly(ADP-ribosyl)ation, plays a critical role in DNA repair [10]. However, when massive damage of DNA occurs, the associated extensive activation of PARP are thought to lead depletion of nicotinamide adenine dinucleotide (NAD), which is the donor of ADP-ribose group. And in efforts to resynthesize NAD, ATP is also depleted resulting in cell death [6,18,19].

The present study tested the hypothesis that poly(ADP-ribosyl)ation and apoptosis contributes to cellular degeneration within the striatum following administration of 3-NP. As in previous reports [2,15], intraperitoneal administration of 3-NP led to selective degeneration within the striatum. Our study showed that enhanced poly(ADP-ribosyl)ation was detected only in the striatal lesions. Our results strongly suggest the involvement of poly(ADP-ribosyl)ation in the development of the striatal lesion after metabolic impairment by 3-NP.

With regard to the apoptosis related gene expression, the ratio of Bcl-2 to Bax in each cells may determine survival or death following apoptotic stimulus [11]. Bax might function as a death effector molecule that is neutralized by Bcl-2. Bcl-2 might simply be an inert handcuff that disrupts the formation of Bax homodimers. Alternatively, Bcl-2 could possess a biochemical function that is diametrically opposed to Bax, or might function as a death repressor molecule that is neutralized by competition with an inert Bax molecule.

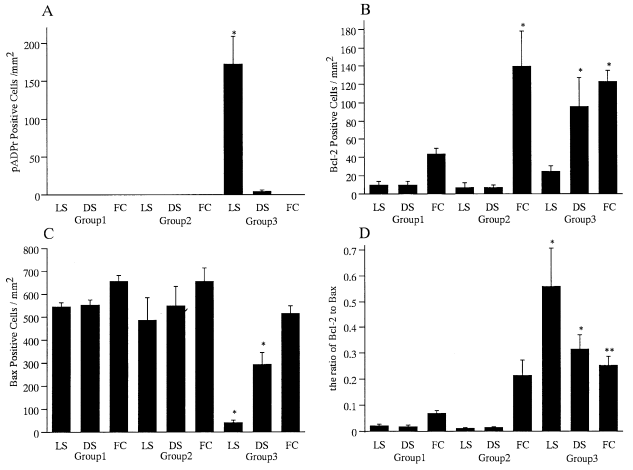

Fig. 4. The number of poly(ADP-ribose)-IR (A), Bcl-2-IR (B), Bax-IR cells (C), and the ratio of Bcl-2 to Bax immunoreactivity (D), in the left lateral striatal lesion (LS), dorsocentral striatum (DS) and frontal cortex (FC) of the animals sacrificed in each group. Data are represented as the mean ± SEM; $n = 8$ animals/group. Values different from control group by $^{*}P < 0.01$, $^{**}P < 0.05$.

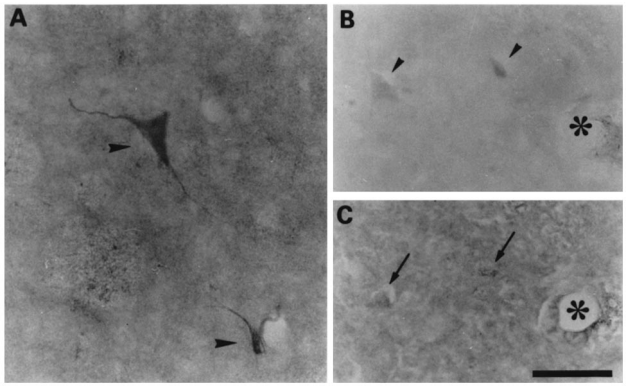

Fig. 5. Photomicrographs of the immunostaining of poly(ADP-ribose) (A,B), neuron specific enolase (NSE) (C) in the lateral striatum of adjacent sections (B,C) of group 3. Poly(ADP-ribose) or NSE-IR cells are indicated as arrow heads or arrows. Asterisk indicates the lumen of an intraparenchymal vessel. Scale bar, 100 μm.

Either way, the capacity of Bax and Bcl-2 to compete for one another via heterodimers suggests a reciprocal relationship in which Bcl-2 monomers or homodimers favor survival and Bax homodimers favor death [11]. Our study showed that the number of Bcl-2-IR cells and the ratio of Bcl-2 to Bax increased outside the lateral striatal lesion where most neurons survive. The increase in the ratio of Bcl-2 to Bax but no change in the number of Bcl-2-IR cells in the lesion may mainly reflect the drastic decrease in the number of the Bax-IR cells due to neuronal loss. Taken together, our results suggest the involvement of poly(ADP-ribosyl)ation and the change of the apoptosis related gene expression in 3-NP neurotoxicity.

Further studies are necessary to elucidate the precise mechanisms of poly(ADP-ribosyl)ation and apoptosis induced by 3-NP.

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