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Expression of cell-cycle-related proteins and excitoxicity

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Summary. Previous work from our laboratory has suggested the functional contribution of p53 to the cascade of events triggered by excitatory amino acids and leading to cell death in primary neurons. Here we show that this paradigm can be extended to cortical neurons treated with NMDA. We found that exposure of the cells to either 300 μM or 2 mM NMDA induced an enhancement of p53 protein levels which was already significant at 60 min after the lesion, while very low staining of the protein was observed in untreated cells. The effect was time- and concentration-dependent, reaching the maximal induction at 3 h. NMDA treatment also resulted in an increase of gadd45 protein levels which was evident in both treatment at 3 h, the time when p53 was maximally induced.

Our data give further evidence suggesting that a repertoire of events typical of proliferating cells is activated in degenerating neurons.

Keywords: Cortical neurons – Cyclin-dependent kinase – Excitotoxicity – Glutamate – Neurodegenerative diseases – Tumour suppressor gene – Gadd45

Abbreviations: NMDA, N-Methyl-D-Aspartate, ROS, Reactive oxygen species; EEA, excitatory amino acids, Gadd45, growth arrest and DNA damage inducible gene; 8-OHdG, 8-hydroxyl-2′-deoxyguanosine

Introduction

There are increasing evidence that cell cycle-associated proteins are re-expressed in neurons committed to death. This process may be as consequence of a variety of insults, including excitotoxins, hypoxia and ischemia, loss of trophic support, or beta amyloid peptide (see Copani et al., 2001, as review). In some of these conditions, events that are typical of the mid-G1 phase, such as cyclin dependent kinase 4/6 activation, are required for the induction of neuronal death. In other cases, the cycle may proceed further and recruit steps that are typical of the G1/S transition for death to occur. Finally, there are conditions in which cell cycle proteins are re-expressed but do not contribute to neuronal death. We have recently hypothesized that cell cycle signalling may became a mandatory component of neuronal death when other mechanisms are not enough for neurons to reach the threshold for death. According to this view, death threshold is set by the extent of DNA damage and the ability of the cell to repair such alteration.

In this paper we summarize recent data in this particular topic and show further evidence for the involvement of cell-cycle-related proteins in the intracellular mechanisms activated by ionotropic glutamate receptor leading to cell death.

Materials and methods

Cell culture

Primary cultures of cortical neurons were prepared from fetal mice at 14–15 d of gestation. Briefly, cells were plated onto poly-l-lysine-coated dishes and cultured for 10 days in vitro (DIV) in a Minimal Essential Medium supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum and 2 mM glutamine, at the density of 1.5×10^5 cells/cm^2.

Evaluation of neuronal death

Neurotoxicity was evaluated in cortical neurons at DIV 10 following the exposure of the cells to 0.3 or 2.0 mM N-methyl-D-Aspartate (NMDA). The concentration of NMDA used in the present study were sub-maximally effective in terms of cell death and were chosen on the bases of the results from a series of previous experiments using different concentrations of NMDA, ranging from 50 μM 1.0 mM. Apoptotic cells were evaluated morphologically by the rapid onset of pyknotic and shrinking nuclei detected by DAPI fluorescent stain. Briefly, at different time points after NMDA treatment cells were fixed in 4% paraformaldehyde, incubated for

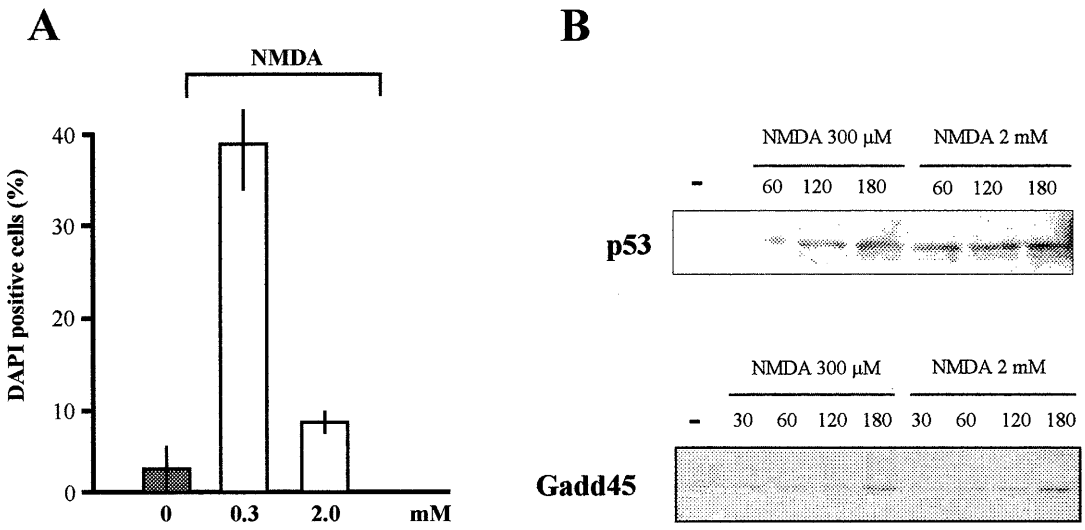

Fig. 1. Effects of different concentrations of NMDA on apoptosis (**A**) and p53 and Gadd45 expression (**B**) in cortical neurons. Panel **A**. Cells were exposed to either 0.3 mM or 2.0 mM NMDA. Detection of DAPI staining was performed 24 h after the treatment. Values are expressed as % of DAPI positive cells over total and represent the mean ± S.E.M. of three separate experiments. Panel **B**. Induction of p53 and Gadd45 in primary cortical neurons exposed to 300 μM or 2.0 mM NMDA: representative Western blot analysis. Cells were exposed to NMDA as described in the method section. Protein extracts were prepared from cells at different times after the addition of NMDA as indicated on the top on the figure. Samples were electrophoresed, transferred to nitrocellulose paper and immunoblotted with anti-p53 or anti Gadd45 antibody. Images are from a representative experiment; similar results were obtained in four different experiments from three separate cell preparations

15 min with (5 mg/ml) DAPI and then observed under fluorescence optics.

Western blot analysis

Cells were harvested in 100 μl of lysis buffer containing 50 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM phenyl methyl sulphonyl fluoride, 0.5 μg/μl leupeptin, 5 μg/μl aprotinin, 1 μg/ml pepstatin. Samples were sonicated and centrifuged at 15,000 g for 30 min at 4°C. The resulting supernatants were isolated and protein content determined by a conventional method (BCA protein assay Kit, Pierce, Rockford, IL). 30 μg of total proteins were electrophoresed on 12% SDS-PAGE, and transferred to nitrocellulose paper (Schleicher and Schuell, Dassel, Germany). Filters were incubated at 4°C overnight with the polyclonal anti-p53 antibody or the polyclonal anti-GADD45 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 3% non-fat dried milk (Sigma). The secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and a chemiluminescence blotting substrate kit (Boehringer, Mannheim, Germany) were used for immunodetection.

Results

Previous work from our laboratory has suggested the functional contribution of p53 to the cascade of events triggered by excitatory aminoacids and leading to cell death in primary cerebellar neurons (Uberti at al., 1998, 2000a, 2000b). Here we show that this paradigm can be extended to cortical neurons treated with NMDA. Two concentrations of NMDA, i.e. 300 μM and 2.0 mM, were used to induce cells death. Indeed, both experimental neurotoxic paradigms result in cell death that can be easily detected 18–24 h after the lesion by a variety of techniques, including LDH release or propidium-iodide staining (data not shown). However, sign of apoptosis as detected by DAPI staining were found only when cells were exposed to 300 μM NMDA (Fig. 1, panel A). These results indicated that exposure of cortical neurons to different concentrations of NMDA results in different types of cell death.

Exposure of the cells to either 300 μM or 2.0 mM NMDA induced an enhancement of p53 protein levels which was already significant at 60 min after the lesion, while very low staining of the protein was observed in untreated cells. The effect was time-dependent, reaching the maximal induction at 3 h. Since under similar experimental conditions up-regulation of p53 is associated with an increased DNA binding activity, we evaluated the possibility that, in this paradigm, p53 induction could result in transcription of specific downstream p53-dependent genes. Thus, we investigated on the involvement of gadd45, a p53 target gene which is recognized as an important factor regulating cell cycle arrest and activating DNA damage repair systems.

Protein extracts from cortical neurons treated with NMDA were analyzed by Western blot using an anti-

Gadd45 antibody. We found that both NMDA treatments resulted in an increase of gadd45 protein levels which was evident at 3 h, the time when p53 was maximally induced.

Representative results from Western blot analysis with anti-p53 and anti gadd45 are shown in Fig. 1, panel B.

Discussion

The possible link between excitotoxicity and the induction of cell cycle-related factors has been matter of extensive investigation during the last 5–10 years and it is now supported by several *in vivo* and *in vitro* data.

In particular, *in vitro* studies have elucidated the role of p53 in apoptosis induced by EAA. Exposure of primary cultures of cerebellar granule neurons to neurotoxic concentrations of glutamate was found to induce a significant, short-lasting increase of p53 expression (Uberti et al., 1998; Grilli and Memo, 1999). Transcriptional activity of the over-expressed p53 was demonstrated by an increased p53 DNA binding activity (Uberti et al., 1998), and the concomitant enhancement of waf1/cip1 kinase inhibitor p21 (Uberti et al., 2000b). Finally, the direct correlation between p53 expression and glutamate-induced apoptosis in cerebellar granule cells, has been suggested by the finding that under the same experimental conditions, a p53 specific antisense oligonucleotide prevented both glutamate-induced p53 expression and apoptosis (Uberti et al., 1998). Relevance of p53 per se in modulating cell viability has been suggested by Xiang et al. (1996) who demonstrated that restoring p53 expression to p53-deficent neurons by adenovirus-mediated transduction is sufficient to promote cell death. In primary cultures of cerebellar granule cells, exposure to the ionotropic glutamate receptor agonist kainate induced a time- and concentration-dependent increase of cyclin D1 expression and apoptosis. The observation that cyclin D1-increased expression was prevent by non-NMDA receptor antagonist treatment, suggests its involvement in excitotoxic receptor-mediated apoptosis (Giardina et al., 1998). We have further extended this concept here by demonstrating the induction of p53 and Gadd45 in primary cortical neurons exposed to NMDA.

Primary cortical neurons may respond to NMDA in a concentration-dependent manner. In fact, treatment of these cells with both low (300 μM) and high (2.0 mM) concentrations of NMDA results in a marked reduction of cell viability. However, signs of apoptosis can be detected only in cells exposed to low NMDA concentrations. Since both treatment induced p53 expression, one may speculate that over-activation of NMDA receptors is generally associated with the induction of an intracellular apoptotic pathway which may rich or not the final execution.

The mechanism(s) by which glutamate induces activation of cell cycle related factors and apoptosis is not clear. We hypothesize that over-stimulation of ionotropic glutamate receptors, possibly by generating oxygen free radicals, may induce DNA damage. A single and/or double-strand DNA breaks caused by excitotoxicity has been suggested by numerous studies (Didier et al., 1996; Liu et al., 1996; Chen et al., 1997). Very recently, Lan et al. (2000) have shown that systemic injection of kainate significantly increased levels of 8-hydroxyl-2′-deoxyguanosine (8-OHdG) in the thalamus, amygdala/piriform cortex and hippocampus. 8-OHdG is an accurate marker of oxidative DNA damage, thus it is most commonly formed by the actions of reactive oxygen species (ROS) on guanine (Park et al., 1989; Dizdaroglu, 1991; Kaur and Halliwell, 1996). A link among excitotoxicity, ROS generation, and DNA damage has been suggested by Hirata and Cadet., (1997) who demonstrated that kainate-induced DNA damage, as measuring by nick translation technique, was attenuated in CuZn superoxide dismutase transgenic mice.

In conclusion, we suggest that, similarly to proliferating cells, postmitotic neurons may respond to (EAA-induced) DNA damage by activating a cascade of events involving DNA damage sensors and repairing factors. In this regard, it has been found that the expression of the DNA mismatch repair factor MSH2, which is a p53 downstream gene functioning in recognition and repair of a several types of DNA damage (Kolodner and Marsischky, 1999), is significantly increased in primary cultures of cerebellar granule cells after glutamate exposure (Uberti et al., 2000b) as well as in CA3 hippocampal neurons after kainate treatment (Belloni et al., 1999).

The increased expression of gadd45 in response to NMDA treatment may further support this view. In fact, evidence suggests that gadd45 may play a role in growth arrest and DNA damage repair (Sheikh et al., 2000). In particular, gadd45 has been shown to interact with products of two other p53-regulated genes, p21 and PCNA and has been implicated in nucleotide excision repair Moreover, gadd45 may promote

G2/M arrest, possibly by interacting and inhibiting cdc2 kinase activity (Zhan et al., 1999). Such arrest will allow cell to activate different machineries for DNA damage repairing. Finally, it is interesting to note that induction of gadd45 has been also found *in vivo* after focal ischemia (Jin et al., 1996).

Although the exact function of gadd45 in EAA-induced cell death remains unclear, here we report further evidence suggesting that a repertoire of events typical of proliferating cells is activated in degenerating neurons.

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