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Induction of p53 in the glutamate-induced cell death program

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Summary. Fifteen minute exposure of primary cultures of cerebellar granule cells to micromolar concentrations of glutamate results in apoptotic cell death. Among the intracellular events triggered by glutamate, we identified two transcriptional factors, i.e. the p50 member of the NF-κB family and the tumor suppressor phosphoprotein p53, that are apparently linked by a sequential trascriptional program. We found that pretreatment of the cultures with aspirin (ASA), which inhibits NF-κB activation, resulted in a complete prevention of glutamate-induced p53 immunoreactivity. The same results were obtained pretreating the cells with a specific p53 antisense oligonucleotide. Both ASA and p53 antisense abolished glutamate-induced apoptosis. We also found that two other proteins, the cyclin dependent kinase inhibitor p21 and DNA mismatches repair MSH2, whose encoding genes are well known target of p53, were upregulated by glutamate. On these bases, we propose NF-κB, p53, p21 and MSH2 as relevant contributors of the glutamate-induced pro-apoptotic pathway.

Keywords: Amino acids – Cerebellar neurons – Cyclin-dependent kinase – Excitotoxicity – Glutamate – Neurodegenerative diseases – Tumour suppressor gene

Abbreviations: ASA, aspirin; cdk, cyclin-dependent kinase; MSH2, MutS Homolog 2; NF-κB, Nuclear Factor – kappa B

Introduction

Glutamate is the most abundant excitatory neurotransmitter in the brain; however, under certain undefined conditions, it may become a potent excitotoxin. Its contribution to the neurodegeneration associated with acute and chronic neurodegenerative diseases, is widely recognized (Lipton and

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Rosenberg, 1994). In particular, brain damage through excitotoxicity has been closely associated to acute conditions like stroke, trauma, hypoglycemia, but also to epilepsy and amyotrophic lateral sclerosis. In addition, a contribution of excitotoxicity to chronic and progressive neuropathologies like Alzheimer's and Parkinson's diseases has been suggested (Lipton and Rosenberg, 1994). Incidentally, signs of apoptosis have been associated, at least in part, with the neuronal death found in many of these diseases, including ischaemia, Parkinson's Disease and Alzheimer's Disease (Robbins et al., 1985; Duguid et al., 1989; Mazzarello et al., 1992; Boerrigter et al., 1992; Portera-Cailliau et al., 1995; Liu et al., 1996).

Several models of neurons in culture have been extensively used to unravel the molecular events triggered by glutamate and leading to cell death as well as to develop a variety of pharmacological compounds able to counteract excitotoxicity. Among them, there is the primary culture of rat cerebellar granule cells, where a brief pulse of glutamate, through activation of the NMDA-type of glutamate receptor, induces cell death (Choi, 1988). There is an emerging consensus that glutamate, through the activation of specific glutamate receptor subtypes, activates a series of genes whose products trigger intracellular events leading eventually to neuronal cell death. Nevertheless, the relative functional contribution of the individual gene products to the glutamate-induced neuronal death has not been completely clarified. It has been suggested that glutamate, possibly by increasing intracellular calcium concentration and/or oxygen free radicals production, may activate a restricted number of transcription factors which in turn amplify the signal by recruiting other genes to dictate specific transcriptional programs.

In the present paper, we report some recent data obtained in our laboratory with the aim both to identify and to characterize the mechanism of action of cytosolic and nuclear proteins known to be involved in cell cycle regulation as well as in promoting degeneration and apoptosis of neurons. Different molecules have been taken into consideration, including members of the NF-κB/Rel transcription factor family, the tumour suppressor protein p53, the cyclin-dependent kinase (cdk) inhibitor p21, and the DNA mismatch repair protein MSH2. These proteins are apparently linked by a sequential transcriptional program which suggests the existence of an intracellular pathway responsible for the induction and progression of neuronal apoptosis.

Material and methods

Cell culture

Primary cultures of cerebellar granule cells were prepared from 8-day-old Sprague-Dawley rat pups as previously described (Uberti et al., 1998). Briefly, cells were plated onto poly-l-lysine-coated dishes and cultured for 12 days in vitro (DIV) in basal Eagle's medium containing 10% heat-inactivated foetal bovine serum, 2 mM glutamine, 50 μg/ml gentamicin and 25 mM KCl, at the density of 1.5×10^5 cells/cm^2. Cytosine arabinoside (100 μM) was added to the cultures 18 h after seeding to prevent non-neuronal cell proliferation.

Evaluation of neuronal death

Neurotoxicity was evaluated in cerebellar granule cells at DIV 12 following the exposure of the cells to glutamate (100 μM). Culture-conditioned media of cerebellar granule cells were collected and the cells were washed once with 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 exposed to glutamate for 15 min. After this period, cells were washed three times with Locke's solution containing 1 mM $MgSO_4$ and returned to the original culture-conditioned media. The apoptotic cells were identified using TUNEL method to label specifically the 3′-hydroxyl termine of DNA strand breaks. For TUNEL staining, all reagents were part of a Kit (Boehringer Mannheim), and the procedures were performed according to the manufacturer's instructions. The quantitative analysis of TUNEL positive nuclei were performed on three different fields per sample. The statistical significance of differences between the values was made by one-way analysis of variance followed by Student's t test. Data are presented as the mean ± SEM of at least three experiments performed in triplicate.

Immunocytochemistry

Cultures were fixed for 30 min in 50 mM PBS containing 4% paraformaldehyde. Immunostaining was performed according to Uberti et al. (1998) The antibodies used were: a polyclonal anti-p53 antibody (R19), from Santa Cruz Biotechnology, used at 1:500 dilution; a polyclonal anti-MSH2 antibody (provided by Dr. J. Jiricny) used at 1:500 dilution; a monoclonal anti-p21 antibody (Santa Cruz Biotechnology) used at 1:1,000 dilution; and a polyclonal anti-p50, used at 1:200 dilution (Grilli et al., 1996a). A semiquantitative study of immunoreactivity was carried out using the Magiscan Image Analysis System, designed and made by Joyce-Loebl Ltd (London, UK). The General Image Analysis Software (Genias) provided by Joyce-Loebl as standard with Magiscan gave us access to the image processing and analysis function of Magiscan. The microdensitometric analysis was performed by measuring the following parameters in each cell body: i) integrated optical density (ID), i.e. the sum of optical density for each pixel in the cell area considered; and ii) area, i.e. the sum of pixels over the image of the cell pointset. This evaluation allows subtraction of background to normaliza values from different samples. The ratio ID/area from each cell was processed for the statistical analysis. The statistical significance of differences between the values was made by one-way analysis of variance followed by Student's t test. Data are presented as the mean ± SEM of at least three experiments performed in triplicate.

Results

Induction of p50 and p53 in glutamate-induced cell death

Previous works have suggested the functional contribution of p53 and NF-κB proteins to the cascade of events triggered by excitatory aminoacids and leading to cell death in primary cultures of rat cerebellar granule neurons (Grilli et al., 1996b; Uberti et al., 1998; Grilli and Memo, 1999). We then investigated the role of a specific member of the NF-κB family, namely the NF-κB1 or p50 subunit, in the glutamate-induced apoptosis. By means of specific antibody, p50 appeared to be homogeneously present in cerebellar granule cells. To evaluate the induction of this protein by neurotoxic concentrations of glutamate, cells were treated with 100 μM glutamate for 15 min and 30 min later processed for immunocytochemical analysis. This well

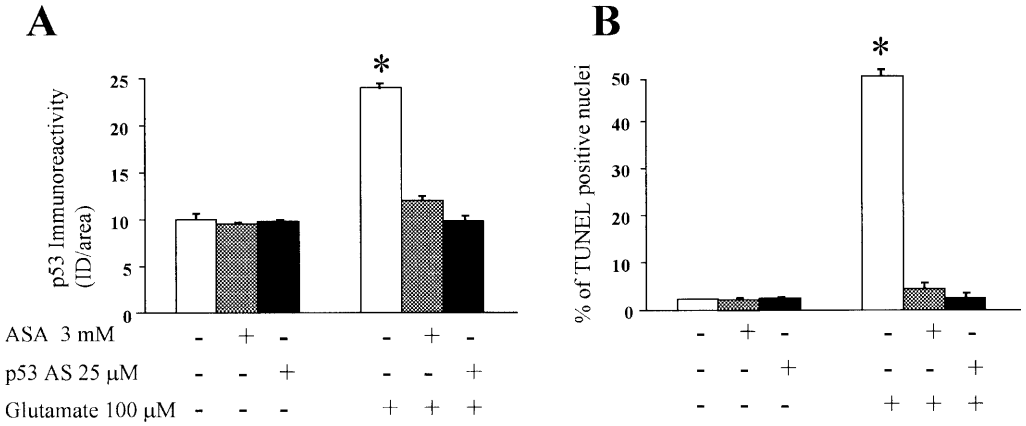

Fig. 1. Effects of glutamate on cerebellar granule cells, treated with vehicle (open bars), 3 mM ASA (filled bars) or 25 μM p53 antisense oligonucleotide (hatched bars). **A** p53 immunoreactivity, evaluated as ID/area (see method section) was carried out with anti-p53 antibody (R19) 30 min after glutamate pulse. **B** Apoptosis was evaluated as % of TUNEL positive nuclei over total cells counted in at least three different fields per sample. Data represent mean ± SEM of three separate preparations. * $p < 0.01$ vs the corresponding control values

recognized experimental neurotoxic paradigm results in cell death that can be easily detected 18–24 h after the lesion by a variety of techniques (Choi, 1988). Exposure of the cells to glutamate induced an enhancement of p50 immunoreactivity which was already apparent within 30 min after the challange, while very low staining of the protein was observed in untreated cerebellar neurons (data not shown). Pretreatment of the cells with 3 mM ASA completely prevented the glutamate-induced increase of p50 immunoreactivity and DNA binding activity (Grilli et al., 1996b). In the same experimental conditions, we also studied the expression of p53. Cerebellar granule cells constitutively expressed p53, as previously demostrated by Western blot and immunocytochemistry analysis. A significant increase of the protein levels was observed 30 min following glutamate exposure (Fig. 1A). Pretreatment of the cells with 3 mM ASA prior glutamate exposure resulted in a complete prevention of the glutamate-induced enhancement of p53 immunoreactivity (Fig. 1A). According to previous data (Uberti et al., 1998), similar results were obtained by preincubating sister culture dishes with a specific p53 antisense oligonucleotide (Fig. 1A).

Glutamate-induced p53 transcriptional activity

Since under the same experimental conditions upregulation of p53 is associated with an increased DNA binding activity (Uberti et al., 1998), we evaluated the possibility that, in this paradigm, p53 induction could result in transcription of specific downstream p53-dependent genes. Two well characterized p53 genes were taken into consideration: p21 and MSH2 (El-Deiry et al., 1993; Scherer et al., 1996).

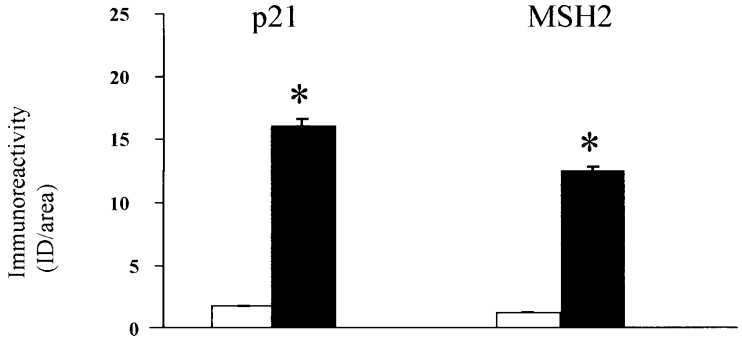

Fig. 2. Effects of glutamate on p21 and MSH2 immunoreactivity in cerebellar granule cells. Immunocytochemistry was carried out with anti-p21 antiboby or anti-MSH2 antibody in control cells (open bars) and 2 h following glutamate pulse (filled bars). Data were expressed as ID/area (see method section). Data represent mean ± SEM of three separate preparations. * $p < 0.01$ vs the corresponding control values

As shown in Fig. 2, cerebellar granule cells costitutively expressed p21 protein, even if at low levels. A significant increase of p21 immunoreactivity become apparent 2 h following glutamate exposure. Untreated cerebellar neurons were also found to be immunoreactive to anti-MSH2 antibody. A marked increase of MSH2 immunoreactivity was observed 2 h after the exposure of the cells to 100 μM glutamate (Fig. 2).

Modulation of glutamate-induced apoptosis

To investigate the possible contribution of p53 and NF-κB to glutamate-induced apoptosis, cerebellar granule cell cultures were pretreated either with ASA or with a specific p53 antisense oligonucleotide before the neurotoxic insult. 3 mM ASA was added to the cultures 5 min before glutamate addition, while 25 μM p53 antisense was added 2 h prior the neurotoxic insult.

TUNEL positive nuclei were observed eight hours following glutamate exposure, suggesting the onset of an apoptotic program. Interestingly, ASA or p53 antisense completely prevented glutamate induced apoptosis (Fig. 1, panel B). Cells treated with ASA or p53 antisense alone exhibited very few scattered TUNEL positive nuclei that were comparable with the control cells (data not shown).

Discussion

In recent years, we have focussed our interest on the role of nuclear proteins that are involved in promoting degeneration and apoptosis of neuronal cells. Most of our studies were carried out in primary cerebellar neurons. This in vitro model offers a morphologically defined system for studying transsynaptic regulation of neuronal gene expression and analyzing the precise temporal sequence of molecular events following stimulation of

specific glutamate receptor subtypes. Exposure of these cells for a brief period of time (15 min) to relatively high concentrations of glutamate (micromolar range) results in cell death of both necrotic and apoptotic types (Choi, 1988; Nicotera et al., 1997). Among the intracellular events triggered by neurotoxic concentrations of glutamate, we identified two transcriptional factors: NF-κB/Rel and the tumor suppressor phosphoprotein p53. Immunocytochemistry and Western Blot analysis demostrated that glutamate upregulates the p50 member of the NF-κB family and p53. Under the same experimental conditions producing cell death, pretreatment of the cultures with ASA, which inhibits NF-κB/rel activation (Yin et al., 1998), or with specific p53 antisense oligonucleotide resulted in a complete prevention of glutamate-induced increase of p53 immunoreactivity. Furthermore, ASA and p53 antisense treatments prevented apoptosis induced by glutamate. These findings suggest the existence of a trascriptional program activated by glutamate receptor stimulation in which p53 and specific members of the NF-κB family are active contributors (Grilli and Memo, 1999).

Numerous *in vitro* studies have demonstrated that diverse neurotoxic and pro-apoptotic stimuli including high concentrations of glutamate (Grilli et al., 1996a), β amyloid (Kaltschmidt et al., 1997), cytokines (Kessler et al., 1993; Talley et al., 1995), glycated tau (Yan et al., 1995), H_2O_2 (Whittermore et al., 1994), and glucose deprivation (Tong and Perez-Polo, 1995), are potent activators of NF-κB in neuronal cells (Abbadie et al., 1993; Grimm et al., 1996; Lin et al., 1995). Moreover, activated NF-κB has been shown in brains of patients affected by a number of neurological diseases in which apoptosis plays a relevant role (Atwood et al., 1994; Dollard et al., 1995; Hunot et al., 1997; Yan et al., 1996). NF-κB activity was also found induced in animal models of neurodegeneration like ischemia (Clemens et al., 1998), head trauma (Yang et al., 1995), Huntington's disease (Quin et al., 1998), and experimental allergic encephalitis (Kaltshmidt et al., 1994).

Similarly, a series of recent papers have contributed to unravel the active contribution of p53 to neurodegeneration (Chopp et al., 1992; Eizenberg et al., 1996; Li et al., 1994; Sakhi et al., 1994; Xiang et al., 1996; Uberti et al., 1998). In particular, systemic injection of kainic acid, a potent excitotoxin that produces seizures associated with a defined pattern of neuronal cell loss, induces p53 expression in neurons exhibiting morphological signs of damage (Sakhi et al., 1994). Moreover, Morrison et al. (1996) found that systemic injection of kainic acid to p53 gene deficient mice does not result in neuronal cell death. Hirata and Cadet (1997) have further corroborated the relevance of p53 in promoting neuronal cell death program showing that homozygous p53-knockout mice are protected against neurotoxicity induced by methamphetamine. Our contribution was the demostration of a direct activation of NF-κB and p53 in response to a pro-apoptotic stimulus.

Then, we analysed the expression of two p53 downstream target genes: p21, which codes for an inhibitor of cdk complexes, and MSH2, which codes for a protein involved in the recognition and repair of a specific type of DNA damage (Palombo et al., 1994). We found that primary cerebellar neurons expressed p21 at very low levels in basal conditions. However, very soon after

a brief exposure of the cells to glutamate, p21 expression was dramatically enhanced. Exposure of cerebellar granule cells to neurotoxic concentrations of glutamate resulted also in a marked increase of MSH2 immunoreactivity. Up to date, very little is known about the role of MSH2 in the brain (Belloni et al., 1997; 1999). It could be speculated that MSH2 may act as sensor of DNA integrity. DNA repair is in fact intimately linked with cell cycle progression, and apoptosis is recognized as a physiological response to DNA damage.

In conclusion, we suggest that glutamate, possibly by increasing intracellular calcium concentration and/or oxygen free radicals production, may activate a restricted number of transcription factors which in turn amplify the death-inducing signal by recruiting other genes to dictate specific transcriptional programs. A hierarchy of intervention is likely to occur. We propose that NF-κB proteins are among the initial orchestrators of the glutamate-induced apoptotic program. Downstream NF-κB-activation is the transcription factor p53. One of the consequence of the increased transcriptional activity of p53 is the up-regulation of p21 and MSH2. On these bases, we propose that NF-κB, p53, p21 and MSH2 could be relevant contributors to the glutamate-induced neuronal apoptosis. The fact that these proteins are also involved in cell cycle regulation supports the hypothesis that aberrant expression of mitotic proteins participates in neuronal cell death program.

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