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Review

Possible role of NF-κB and p53 in the glutamate-induced pro-apoptotic neuronal pathway

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Abstract

Apoptosis is now recognized as an important component in many progressive and acute neurodegenerative diseases. Extracellular signals and intracellular mechanisms triggering and regulating apoptosis in neuronal cells are still a matter of investigation. Here we review data from our and other laboratories with the aim to elucidate the nature of some proteins which are known to be involved in cell cycle regulation as well as in promoting degeneration and apoptosis of neurons. The following molecules will be taken into consideration: NF-κB, p53, p21 and MSH2. These proteins are activated by neurotoxic experimental conditions which involve the stimulation of selective receptors for the excitatory aminoacid glutamate. Thus, we hypothesize their contribution to an intracellular pathway responsible for the glutamate-induced neuronal death. Identification of such mechanisms could be relevant for understanding the apoptosis associated with various neurodegenerative diseases as well as for developing novel strategies of pharmacological intervention.

Keywords: cerebellar neurons; cyclin-dependent kinase; excitotoxicity; glutamate; neurodegenerative diseases; tumour suppressor gene

Abbreviations: cdk, cyclin-dependent kinase; CNS, central nervous system; MSH2, mutS homolog 2; NF-κB, nuclear factor - kappa B

Introduction

Apoptosis of neuronal cells has been suggested to be the result of aberrant cell cycle control. This hypothesis stands on several independent lines of reasoning that foster the notion of a link between cell cycle and apoptosis. First, apoptosis is frequently observed in highly proliferative cells such as embryonal cells, hematopoietic cells, and neoplastic cells. Second, some of the morphological features observed during apoptosis, including cell rounding, nuclear envelop breakdown, chromatin condensation, and nuclear lamina disassembly, occur during mitosis. Third, activation of a number of genes that mediate the transition of cells from quiescent to proliferative state is associated with apoptosis. In this regard, it has been shown that apoptosis of neurons may be activated in response to molecular events that lead to transformation of dividing stem cell populations. The relationship between transformation and neurodegeneration originally proposed by Heintz,[1] was based on accumulating evidence which suggest that neoplastic lesions that generate uncontrolled cell proliferation can also act as potent triggers of apoptosis of terminally differentiated neurons. According to this theory, terminal differentiation of neurons is associated with the suppression of cell division program but, if these cells are forced to reenter the cell cycle, they activate an aberrant cell cycle program that irreversibly will lead to cell death. Thus, it is allowed to speculate that some cell cycle components are implicated in at least some forms of neuronal apoptosis.

The present paper will review some recent data obtained in our and other laboratories 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 will be 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. We hypothesize that one of the orchestrators of such intracellular program may be the excitatory aminoacid glutamate. Glutamate is the most abundant excitatory neurotransmitter in the brain. However, under certain conditions, it may become a potent excitotoxin.[2] 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.[3] 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.[4–9]

NF-κB/Rel proteins

Initially regarded as a B cell-specific transcription factor,[10] NF-κB was soon demonstrated to be present in an inducible form

in most eukariotic cells where, upon stimulation by a wide variety of stimuli, it translocates to the nucleus and regulates transcription of target genes.[11] Along the years, NF-κB system has been recognized as a family of transcription factors mediating the rapid and coordinated induction of genes in response to external, primarily pathogenic stimuli. An inappropriate regulation of NF-κB-mediated transcription has been in turn associated with pathological conditions including acute inflammatory reactions, toxic/septic shock, acute phase reactions, atherosclerosis, radiation damage, viral replication, miocardial infarction, cancer and several neuropathologies.[12] The finding that proteins belonging to the NF-κB family are also present in the central nervous system (CNS) is relatively recent.[13] Of particular interest with regard to the CNS location and function of NF-κB/Rel proteins is the demonstration that unlike most of the cells in periphery, except for B lymphocytes, a high constitutive NF-κB activity can be present in neurons. For example, nuclear NF-κB specific immunostaining appeared to be present only in specific cellular subsets in rat cortex and hippocampus, while in other cell populations it is restricted to cytoplasm. In light of this observation it has been suggested that the activation state of NF-κB might correlate with neuronal activity and that the modulator might be involved in regulation of cellular antioxidant program in metabolically very active neurons.[13,14] Furthermore, neuronal NF-κB inducible activity has been demonstrated not only in neuronal bodies but also in synapses[15–17] and in post-synaptic densities[18] to suggest its activity as a crucial neuronal messenger carrying synaptic information to the nucleus. In the brain, NF-κB/Rel protein expression is not restricted to neurons. These transcription factors are in fact also present, as inactive cytoplasmic form, in glial cells, including primary astrocytes, astrocytoma cell line, microglia, and Schwann cells.[19–21]

Still limited is the knowledge of the nature of the signals that trigger NF-κB activation within the CNS as well as of the target genes whose function may be relevant in brain physiology and/or pathology. Particularly interesting and relevant to our discussion is the fact that a growing body of evidence is accumulating for a specific activation of NF-κB proteins in both neuronal and non-neronal cells in neuropathologies associated with etiologically unrelated conditions. Activated NF-κB has been shown in brains of patients affected by a number of neurological diseases in which apoptosis play a relevant role. The list includes Alzheimer's disease,[22] Parkinson's disease,[23] AIDS-Dementia complex[24] and encephalitis.[25] Increased NF-κB activity may also be associated with Ataxia Teleangectasia.[26] Moreover, NF-κB activity is induced in animal models of neurodegeneration like ischaemia,[27] head trauma,[28] Huntington's disease,[29] and experimental allergic encephalitis.[20]

Numerous *in vitro* studies have demonstrated that diverse neurotoxic and pro-apoptotic stimuli like high concentrations of glutamate,[30] β amyloid,[31] cytokines,[32,33] glycated tau,[34] H_2O_2,[35] and glucose deprivation,[36] are potent activators of NF-κB in neuronal cells.[37–39] On the other hand, other *in vitro* studies have suggested that NF-κB activation may induce defence mechanisms against neuronal apoptosis.[40–42] Understanding the contribution of NF-κB mediated transcription to neuronal death and/or survival pathways in neurological diseases represents a recently raised debate with important pharmacological implications. The relative contribution of NF-κB-mediated transcription to either survival or degeneration is likely to depend on several variables including the composition of inducible NF-κB complexes, kinetics of activation, concomitant availability of other transcription factors and intrinsic metabolic and genetic differences between neuronal phenotypes or, within the same cell, the nature and the intensity of the activating stimulus.[43–46] More knowledge is also required to understand the relative functional contribution of constitutive *versus* inducible NF-κB activity in gene transcription. Interestingly, it has been recently shown that partial inhibition of constitutively activated NF-κB in fibroblasts from Ataxia Teleangectasia patients results in decreased radiation-induced apoptosis, while inhibition of NF-κB activation in normal cells leads to increased apoptosis.[47] These data can be related to those showing that suppression of steady-state, but not stimulus-induced NF-κB activity inhibits alphavirus-induced apoptosis.[46] Crucial questions arise from these observations. Are the genes 'constitutively' activated by NF-κB cell-specific? Also, do constitutive and inducible NF-κB proteins activate the same transcriptional program? Definitely, to distiguish between pro- and anti-apoptotic pathways triggered by NF-κB activation in response to a specific deleterious stimulus in specific cell phenotypes it will be crucial to identify the nature of the genes that are under the control of NF-κB/Rel factors in the CNS. Interleukin-converting enzyme, amyloid precursor protein, c-*myc*, and APO-1/Fas ligand are good candidates as pro-apoptotic, NF-κB target genes.[13,30,48] We have focused our interest on a particularly intriguing gene which is under transcriptional control of NF-κB, the one encoding the tumour suppressor protein p53.

The tumour suppressor protein p53

The tumour suppressor protein p53 is a cell cycle checkpoint protein that contributes to the preservation of genetic stability.[49,50] More recently, it was found that p53 may also play a role in cell differentiation.[51]

The mechanism(s) by which p53 can induce cell cycle arrest and/or apoptosis is still largely unknown. Development of transgenic mice deficient for p53 has gained further insight on the functional role of p53.[52] Interestingly, mice homozygous for p53 null allele appear quite normal, although female-associated defects in neural closure were found at high frequency in p53 null mice embryos.[53] Thus, p53 function appears to be dispensable in many apoptotic processes that occur physiologically during the entire life-span in a large variety of organs and systems, including the brain.

A series of recent papers have contributed to unravel the positive contribution of p53 to neurodegeneration.[54–57] 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.[56] More recently, Morrison *et al*.[58] found that systemic injection of kainic acid to p53 gene deficient mice

do not induce neuronal cell death. Hirata and Cadet[59,60] have corroborated the relevance of p53 in promoting neuronal cell death program showing that homozygous p53-knockout mice are protected against neurotoxicity induced by methamphetamine. Finally, p53 expression has been investigated in Alzheimer's disease. Post-mortem studies show that Alzheimer's disease brains contain higher p53 levels than age-matched controls, but it is still controversial whether the increased p53 expression is linked to apoptosis in glia or neuronal cells.[61–63]

Excitotoxicity and apoptosis: a role for p53 and NF-κB proteins

We have investigated on the role of p53 and NF-κB proteins in cultured cerebellar granule cells in response to neurotoxicity induced by glutamate. Primary cerebellar neurons offer a morphologically defined system for studying transsynaptic regulation of neuronal gene expression and provide the opportunity to analyze the precise temporal sequence of molecular events following stimulation of specific glutamate receptor subtypes. In fact, exposure of these cells for a brief period of time (15 min) to relatively high concentrations of glutamate (micromolar range) results in cell death which includes both necrosis and apoptosis.[64] Among the intracellular events triggered by neurotoxic concentrations of glutamate is the activation of NF-κB/Rel proteins.[30] Functional significance of such effects has, at least in part, been suggested by the finding that under the same experimental conditions, salycilates prevent glutamate-induced neuronal death and, at the same doses, inhibit the glutamate-induced activation of NF-κB DNA binding activity.[65] Our working hypothesis was that NF-κB may indeed contribute to cell death triggered by glutamate by switching on pro-apoptotic target genes. Among the genes that are transcriptionally regulated by NF-κB is the gene encoding the tumour suppressor phosphoprotein p53.[66] We then investigated the role of p53 in the glutamate-induced cell death.

We found that exposure of cerebellar granule cells to a 15 min-pulse of micromolar concentration of glutamate resulted in a significant, short-lasting increase of p53 expression.[67] Previous studies performed in primary neurons have demonstrated that p53 is essential for excitotoxicity[57] and that can be induced by DNA damage.[68] Our novel contribution is the demonstration of a direct link between neurotransmitter receptor stimulation and p53 induction. Measurement of p53 mRNA levels suggested that treatment of the cells with glutamate results, at least in part, in an increased p53 gene transcription. Furthermore, p53 overexpression was found to be associated with increased p53 DNA binding activity, suggesting an enhanced p53 transcriptional activity. To further support this finding, exposure of cerebellar neurons to glutamate resulted in the induction of at least two gene products that are suggested to be regulated at transcriptional level by p53, p21 and MSH2.

$p21^{CIP1/WAF1}$, an inhibitor of cdk complexes, is a well characterized transcriptional target of p53. It is induced by DNA damage in a p53-dependent manner and is found associated with various types of inactive cyclin-cdk complexes.[69] Primary cerebellar neurons do express p21 at very low levels in basal conditions. However, very soon after a brief exposure of the cells to glutamate, p21 expression was dramatically increased and this effect lasted for several hours (unpublished data). A link between p53, p21 and proteins involved in neurodegeneration has been recently found by Roperch *et al*.[70] who demonstrate that presenilin1 is involved in a series of model systems for p53-dependent and p53-independent apoptosis.

MSH2 is one of the most important proteins involved in the recognition and repair of a specific type of DNA damage that is characterized by pair mismatches.[71] Up to date, very little is known about MSH2 expression in the brain. Using immunohistochemical analysis, we recently found that MSH2 is expressed in selective rat brain regions and it is induced by experimental paradigms of excitotoxicity. Similar results were obtained *in vitro*, using primary cultures of rat cerebellar granule cells. In fact, exposure of the cells to neurotoxic concentrations of glutamate resulted in a marked increase of MSH2 expression.[72]

The role of p53, p21 and MSH2 in definitely post-mitotic cells like neurons has not clearly been established. It could be speculated that p53 and MSH2 may act as sensors 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. Moreover, diseases associated with germ-line mutation of genes involved in DNA damage recognition and repair, such as Ataxia Teleangectasia, present signs of neurodegeneration. A further indirect link between neurotoxicity and DNA damage has been provided by Didier *et al*.[73] who showed accumulation of single-strand DNA damage as an early event in excitotoxicity in primary neuronal cultures. Also, this particular DNA damage results in the induction of p53 expression.[74,75]

To evaluate the contribution of p53 expression to glutamate-induced cell death, we took advantage of the antisense strategy. An oligonucleotide complementary to 18

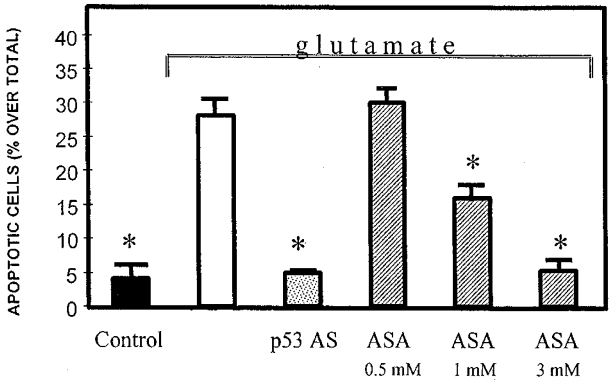

Figure 1 Prevention of glutamate-induced apoptosis in primary cerebellar neurons by a p53 antisense oligonucleotide (p53 AS) and aspirin (ASA). Apoptotic cells were visualized by TUNEL staining. p53 AS (25 μM) was added to the cultures 1 h before the glutamate treatment. ASA, at the concentrations shown in the figure, was added 15 min before glutamate. *, $P<0.01$ *versus* glutamate-induced apoptosis

bases flanking the ATG initiation codon of the p53 gene was synthesized and added to the culture media before treating the cells with glutamate. Blockade of p53 induction, as shown by the prevention of glutamate-induced increase of p53 immunoreactivity, resulted in a complete inhibition of the apoptotic component of the glutamate-induced cell death.[67] The specificity of the effects was proven by the lack of efficacy of the sense oligonucleotide and by the observation that the antisense oligonucleotide treatment did not alter the capability of glutamate of inducing other intracellular processes, including the increase of NF-κB nuclear activity. These data support the existence of an intracellular pathway triggered by glutamate and leading to apoptosis in which p53 induction lies downstream of NF-κB activation. Interference of this pathway at the level of NF-κB, by salycilates, or p53, by specific oligonucleotide antisense, results in a prevention of glutamate-induced apoptosis (Figure 1). In this experimental context, salycilates appear to possess a wider spectrum of neuroprotective activity, being also able to inhibit the glutamate-induced necrosis.

Conclusions

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. 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 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. At the present time, the p53 target genes triggered by glutamate receptor stimulation are largely unknown. We hypothesize that up-regulation of p21 and MSH2 genes, in the experimental paradigm of glutamate-induced neuronal death, is one of the consequences of the increased transcriptional activity of p53. 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. Further studies will be necessary for understanding whether other molecules which are known to be involved in

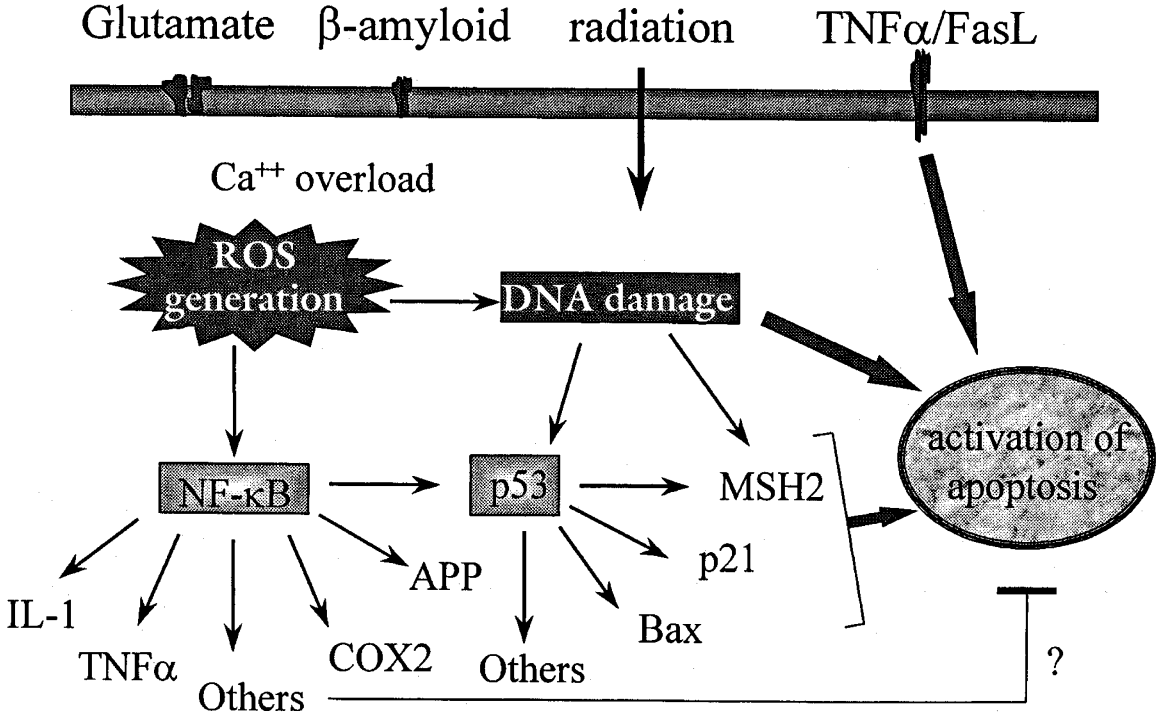

Figure 2 Schematic representation of the intracellular events activated by various apoptotic signals including glutamate, β-amyloid, various types of irradiation and TNFα/Fas ligand. According to our hypothesis, stimulation of ionotropic glutamate receptor by glutamate or exposure to β-amyloid, although with different mechanisms, results in increased intracellular Ca^{2+} concentrations and reactive oxygen species (ROS). These effects may all converge ultimately on NF-κB activation. In the nucleus, NF-κB may induce, together with other transcription factors, a number of genes which could activate intracellular programs leading to either apoptosis or neuroprotection. Factors affecting such choice are at the moment unknown. Among the apoptotic genes activated by NF-κB is p53 which may activate the transcription of other genes, including MSH2 and p21, to progress the apoptotic pathway. Induction of the p53 gene can also occur independently of NF-κB and by direct recognition of damaged DNA. This tentative scheme takes into consideration the occurrence of p53-independent apoptosis which can be triggered by other signals including TNFα/Fas ligand or DNA damage

transformation and degeneration processes, such as members of the bcl-2 family and caspases, are active participants in the NF-κB-induced, p53-mediated pro-apoptotic pathway.

Finally, the glutamate-induced apoptosis of primary neuronal cultures was found strictly p53-dependent. This is not a generalized phenomenon. As an example, apoptosis of cerebellar neurons *in vitro* induced by serum deprivation or low potassium is p53-independent.[76] Moreover, different genotoxic treatments may cause distinct phosphorylation of p53, which may account for the activation of unique pro-apoptotic pathways.[77] In all, these data support the view that different intracellular programs leading to neuronal apoptosis may exist. It could be inferred that the apoptosis that occurs physiologically (i.e. during brain development and ageing) and the apoptosis that is related with neurodegenerative diseases may depend on different molecular participants. This particular topic definitely requires much attention in the development of anti-neurodegenerative drugs acting at transcriptional level. Understanding this cascade of nuclear events may indeed unravel specific targets for pharmacological intervention for those neurological diseases in which specific types of apoptosis play a relevant role.

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