ROLE AND REGULATION OF p53 IN DEPOLARIZATION-INDUCED NEURONAL DEATH

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Abstract—The tumor suppressor gene p53 is a potent transcriptional regulator for genes involved in many cellular activities including cell cycle arrest and apoptosis. In this study, we examined the role of p53 in neuronal death induced by the sodium channel modulator veratridine. We also analyzed the involvement of Ca²⁺, mitochondria and reactive oxygen species in p53 activation. Exposure of hippocampal neurons to veratridine (0.3–100 µM) resulted in a dose-dependent neuronal death, measured 24 h after treatment. p53-Like immunoreactivity, undetectable in neurons under control conditions, was observed in about 25% of neurons, 7 h after veratridine exposure. Treatments that modified the alkaloid-induced Ca²⁺ influx including tetrodotoxin or Ca²⁺ removal, prevented either veratridine-induced cell death or p53 immunoreactivity. Mitochondria were involved in veratridine-induced cell death, as the alkaloid collapsed inner membrane mitochondrial potential in a Ca²⁺ influx dependent manner. Treatments of neuronal cultures with the permeability transitory pore blockers cyclosporin A and bongkrekic acid prevented veratridine-induced p53 immunoreactivity and neuronal death, placing mitochondria upstream of veratridine-induced p53 immunoreactivity. Reactive oxygen species also participated in veratridine-induced neurotoxicity and p53 activation. Antisense knockdown of p53 resulted in a significant increase in neuronal survival after veratridine treatment. This protective effect was maintained on N-methyl-D-aspartate (NMDA) resulted in a dose-dependent neuronal death and that p53 might be a link between toxic stimuli of different types and neuronal death. © 2003 IBRO. Published by Elsevier Ltd. All rights reserved.

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Gene expression is involved in many cell death programs (Kroemer et al., 1995; Henderson, 1996) probably because many cellular responses to environmental changes are mediated by inducible transcription factors (Karin and Smeal, 1992; O’Neill and Kaltschmidt, 1997). The p53 tumor suppressor gene is a sequence-specific transcription factor that activates the expression of genes engaged in promoting growth arrest or cell death in response to multiple forms of cellular stress. However, evidence for transcription-independent pathways for p53-mediated apoptosis is accumulating (reviewed in Ref. Gottlieb and Oren, 1998). In some cell types, p53-induced apoptosis occurs in the absence of gene transcription or protein synthesis (Caelles et al., 1994). Furthermore, inhibitors of protein phosphatases induce p53-dependent apoptosis in the absence of transactivation (Yan et al., 1997; Bennett et al., 1998). Most interestingly, p53 protein from cell-free postnuclear extracts directly mediates activation of effector caspases in transformed fibroblasts that undergo p53-dependent apoptosis after γ-irradiation (Ding et al., 1998).

In the mature nervous system, numerous studies indicate that p53 plays a key role in neuronal death following certain number of insults (Miller et al., 2000; Morrison et al., 2003) including ischemia (Li et al., 1994; Chopp et al., 1992), seizure activity (Sakhie et al., 1994), N-methyl-D-aspartate (NMDA; Uberti et al., 1998), x-irradiation (Morrison et al., 1996; Jordan et al., 1997b) and the death of dentate granule cells after adrenalectomy (Schreiber et al., 1994). In addition, p53-deficient mice show reduced neuronal death after ischemia (Crumrine et al., 1994) and decreased neurotoxicity after kainate administration (Morrison et al., 1996). p53 Overexpression, using an adenoviral vector, in cultured rat hippocampal pyramidal neurons causes widespread neuronal death with features typical of apoptosis without induction of p21, bax, or mdm2. This is consistent with a role for p53 in nerve cell death that is distinct from its actions related to cell cycle arrest (Jordan et al., 1997b).

Veratridine is an alkaloid that binds to neurotoxin receptor site two in voltage-dependent Na⁺ channels (Catterall and Coppersmith, 1981) and prevents its inactivation, causing depolarization of excitable cells by keeping Na⁺ channels open for long periods of time (Bönisch and Keller, 1983). This depolarising effect is accompanied by an increase in both Na⁺ and Ca²⁺ influx (Amy and Kirshner, 1982) that is able to induce release of neurotransmitters from different secretory systems (Ceña et al., 1983). Veratridine has been shown to be toxic to hippocampal neurons and cerebellar granular cells (Schoffelmeer et al., 1985; Pauwels et al., 1991).

Many apoptotic cascades in eukaryotic cells use mitochondria as the key point where diverse death stimuli translate from initiation into execution, irrespective of
whether apoptosis is triggered from inside or outside the cell (for review see Green and Reed, 1998; Peter and Krammer, 1998). Mitochondria undergo critical changes that trigger a common degradative phase. These changes include collapse of the inner transmembrane potential (Δψm), reactive oxygen species (ROS) generation, permeability transition pore (PTP) opening, and mitochondrial swelling with outer membrane rupture in some instances. However, the involvement of p53 in veratridine-induced neurotoxicity has not yet been determined.

In the present work, we have used veratridine-induced neurotoxicity as a useful pharmacological tool to study whether the selective activation of voltage-dependent Na+ channels activates p53 and if Ca2+ influx, mitochondria and oxidative stress are involved in the activation of this transcription factor leading to neuronal death.

**EXPERIMENTAL PROCEDURES**

**Cellular cultures**

Pyramidal neurons were prepared from the hippocampi of Sprague–Dawley fetal rats (Harlam Iberica, Barcelona, Spain) at 17 days of gestation (E17) as previously described (Prehn et al., 1996). Briefly, hippocampi were dissected in Ca2+/Mg2+-free Hanks’ (Celgrò) balanced salt solutions and incubated in 0.1% trypsin ( Worthington Biochemical, Lakewood, NJ, USA) for 15 min. The hippocampi were triturated by aspirating 7–10 times with a Pasteur pipette with a flame-narrowed tip. Cells were plated in Dulbecco’s Modified Eagle medium ( Gibco BRL, Life Technologies, Inc, Grand Island, NY, USA) containing 10% horse serum ( Gibco BRL) on poly-L-lysine ( Sigma, St. Louis, MO, USA) at 15000 cells/cm2. Cells were incubated for 2 days later to support astrocytes attached to the bottom of the culture dish. After incubation, cells were washed twice and incubated for an additional 30 min in K-H solution. The dye was excited using 340 nm and 380 nm excitation filters mounted on a filter wheel under computer control. Excitation and emission (510 nm) were separated by a dichroic mirror (430 nm). The fluorescence ratio F510/F430, independent of the probe concentration, was used as an index of [Ca2+]i changes (Augustine and Neher, 1992).

Mitochondrial transmembrane potential was monitored by measuring rhodamine 123 (Molecular Probes) fluorescence (Duchen and Biscoe, 1992). Neurons were incubated with the dye in K-H solution for 5 min and fluorescence continuously monitored 5 min after washing using an excitation filter of 485 nm and an emission filter of 535 nm (Omega Optical Inc., Brattleboro, VT, USA).

**Cell viability studies**

Cell death was determined using fluorescein diacetate/pro-pidium iodide double staining procedure (Jordan et al., 1997a). Cells were incubated for 45 s at 22–25 °C with 15 μg/ml fluorescein diacetate (Sigma) and 4.6 μg/ml propidium iodide (Molecular Probes, Inc., Eugene, OR, USA) in phosphate buffered saline (PBS) with the following composition (in millimoles/liter): Na2HPO4, 100; NaH2PO4, 100; NaCl, 140; pH 7.4. The stained cells were examined immediately with a standard epifluorescence microscope (Axioptik; Zeiss, Germany). Cells stained with propidium iodide represent dead cells, whereas cells stained with fluorescein represent live cells. A blinded observer counted the number of death and living neurons in five microscopic fields (under 40× magnification) for each coverslip totalling approximately 300–450 cells and their mean was regarded as the representative value for the coverslip. The percentage of neurons surviving was determined and normalized to controls examined in parallel under the same condition. The average relative percent survival from at least three separate experiments for each condition is expressed in the text and figures as the mean±S.E.M. and statistical significance was determined by Student’s t-test.

Analysis of chromatin was performed by staining the neurons with the dye Hoechst 33342. Neuronal cultures were rinsed three times using PBS and then incubated with 1 ng/ml Hoechst 33342 (Molecular Probes) for 20 min at room temperature. Following two rinses with PBS, cell staining was analyzed using a fluorescent microscope.

**Electrophysiological recordings**

Hippocampal neuron membrane potential was determined under current-clamp using the nystatin-perforated patch-clamp recording technique (Horn and Marty, 1988) and an EPC-7 patch-clamp amplifier (List Medicals, Greenvale, NY, USA). K-H solution was used as bath solution and the pipette solution contained (in millimoles/liter): KC1, 40; K2SO4, 50; NaCl, 10; MgCl2, 1; HEPES, 10; glucose, 11; and nystatin, 250 μg/ml; pH, 7.25. After gigaseal formation, neuronal membrane potential was continuously recorded using the program Axtape (Axon Instruments, Foster City, CA, USA). Veratridine (0.3–30 μM) was added using a fast drug application system (DAD system; Adams and List, New York, NY, USA). All recordings were performed at room temperature (22–24 °C) using fire-polished electrodes with an open tip resistance between 2 and 4 MΩ.

**Fluorimetric determinations**

Experiments were performed at room temperature on the stage of a Nikon Diaphot inverted microscope equipped with a 75W Xenon lamp and a Nikon 40×, 1.3 numerical aperture, epifluorescence oil immersion objective. Images were acquired with a CCD camera and analyzed using commercial software (Life Sciences Ltd., England).

To monitor [Ca2+]i, cells were loaded with fura-2 by incubation with fura-2 AM (5 μM; 45 min; 37 °C) in K-H solution. After incubation, cells were washed twice and incubated for another 30 min in K-H solution. The dye was excited using 340 nm and 380 nm excitation filters mounted on a filter wheel under computer control. Excitation and emission (510 nm) were separated by a dichroic mirror (430 nm). The fluorescence ratio F510/F430, independent of the probe concentration, was used as an index of [Ca2+]i changes (Augustine and Neher, 1992).

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Superoxide production was monitored using hydroethidine (Molecular Probes) as described previously (Bindokas et al., 1996). Background was subtracted and fluorescence recorded using an excitation filter of 535 nm and an emission filter of 635 nm (Omega Optical). Frames were recorded every 10 s over a 7 min period. Linear regression of fluorescence data was obtained for each condition and the slope of the best fitting line was taken as an index of O2− production.

**Immunocytochemistry**

Cultures were fixed by incubation at 37 °C for 15 min with 4% paraformaldehyde in 0.1 M PBS (pH, 7.4). After washing three times in 0.1 M PBS, cells were permeabilized using 0.1% Triton X-100 (Merck, Darmstadt, Germany) in PBS for 2.5 min. Coverslips were then incubated for 1 h in blocking media (0.1% Tween 20; 4% BSA in 0.1 M PBS) at room temperature. Incubations with primary antibodies were performed overnight at 4 °C using a monoclonal antibody anti-p53 (DO-1; Santa Cruz, CA, USA; 1:100) diluted in blocking medium. Specific immunolabelling was detected using Histostain-Plus Kit (Zymed, San Francisco, CA, USA).
Oligonucleotide treatment

Both mismatch and antisense p53 oligonucleotides phosphoro-
thioate-labeled were purchased from Proligo (France). p53 Anti-
sense oligonucleotide sequence was the following: 5′-AATCCTC-
cATGACAGTTA corresponding to nucleotide 7 to nucleotide 11 in rat p53 gene sequence. The mismatch oligonucleotide sequence is a random distribution of the sense oligonucleotide. The selected target sequences have relatively low homology with any of the other known cDNA sequences found in the Genebank database, as determined by using the GenePro program (Brain-
bridge Island, WA, USA).

Cultured hippocampal neurones were treated between 7 and 9 days in vitro. The coverslips were removed from the glial feeder
layer, placed face up in a 60 mm tissue culture dish and covered
with a “glia-conditioned” medium (2.5 ml). An aliquot of oligonu-
ucleotide was then added to the medium to reach 1/100 final
concentration. The dish was gently agitated and placed back to
the incubator.

Reagents

Methyl-10,11-dihydro-5-h-dibenzocyclohepten-5,10-imine (dizol-
cipine [MK-801]) was obtained from RBI (Natick, MA, USA) and
1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid tet-
rakis (acetoxymethyl ester) (BAPTA-AM) from Molecular
Probes. Veratridine, catalase, tetrodotoxin (TTX) and vitamin E
were obtained from Sigma. All the other reagents were ob-
tained from commercial sources, being of the maximal available
purity.

RESULTS

Veratridine induces neuronal death and increases p53 protein levels

Hippocampal neurons exposed to increasing concentra-
tions of veratridine (0.3–100 μM), for 30 min, produced
a dose-dependent neuronal death, measured 24 h after
treatment, that reached about 35% and 70% of neurons
for 30 μM and 100 μM veratridine (Fig. 1). Veratridine
(0.3–30 μM) induced membrane potential depolarization
in hippocampal neurons (Fig. 2A), and increments in [Ca2+]i levels (Fig. 2B) in a dose-response manner. As shown in Fig. 3B, neuronal cultures exposed to 30 μM veratridine for 30 min, presented DNA damage hallmarks. After 24 h, chromatin condensation and fragmentation could be observed in veratridine-treated cultures stained with propidium iodide (Fig. 3A).

Several forms of DNA damage have been shown to activate p53 (Sharpless and DePinho, 2002), which has been associated with different insults leading to neuronal death (Morrison et al., 1996; Jordan et al., 1997b; Xiang et al., 1998). The next set of experiments was designed to identify whether p53 was activated during veratridine-in-
duced neurotoxicity. As shown in Fig. 3C, p53 protein was undetectable in these neurons under control conditions, but 7 h after veratridine treatment (30 μM, 30 min), p53-like immunoreactivity was observed in about 25% of neurons (vehicle, Figs. 3D and 4).
Calcium influx is required for veratridine to induce p53 and neurotoxicity

In order to explore the role of Na\(^+\)/H\(^+\) and Ca\(^{2+}\)/H\(^+\) influx as possible mediators for p53 activation, their role in veratridine-induced neuronal death was first analyzed. As shown in Fig. 5, extracellular Na\(^+\) removal (0 Na\(^+\)) or treatment with the sodium channel blocker TTX (10 \(\mu\)M) almost completely prevented veratridine-induced neurotoxic effects. Similar results were found when extracellular Ca\(^{2+}\) was removed (0 Ca\(^{2+}\)) or the hippocampal neurons were loaded with the intracellular Ca\(^{2+}\) chelator BAPTA-AM (10 \(\mu\)M; 60 min). However, treatments with the selective NMDA receptor blocker MK-801 (10 \(\mu\)M) did not protect neuronal cultures against veratridine-induced toxicity (Fig. 5).

Next, we studied whether there was a correlation between the ability of certain treatments to prevent veratridine-induced neuronal death and veratridine-induced Ca\(^{2+}\) influx. Hippocampal neurons showed highly reproducible increases in [Ca\(^{2+}\)]\(_i\) in individual soma in response to two 30 s pulse applications of veratridine (30 \(\mu\)M) applied 5 min apart (peak ratio S₂/S₁ 1.023±0.056; n=49). TTX (peak ratio S₂/S₁ 0.059±0.035; n=15) or 0 Ca\(^{2+}\) (peak ratio S₂/S₁ 0.064±0.071; n=26) blocked veratridine-induced increase in [Ca\(^{2+}\)]\(_i\), while MK-801 had no effect on veratridine-induced increase in [Ca\(^{2+}\)]\(_i\) (peak ratio S₂/S₁ 0.976±0.056; n=49), suggesting that there was a good correlation between Ca\(^{2+}\) influx blockade and neuroprotection.

The role of Ca\(^{2+}\) influx into the neurons in veratridine-induced p53 activation was explored performing immunostaining studies. As it can be observed in Fig. 4, treatments that modified the alkaloid-induced Ca\(^{2+}\) influx like TTX (10 \(\mu\)M) or Ca\(^{2+}\) removal, prevented p53 immunoreactivity, while MK-801, that did not modify Ca\(^{2+}\)-influx, failed to prevent the increase in p53 immunoreactivity, suggesting a link between Ca\(^{2+}\) influx and development of p53 immunoreactivity.

Role of mitochondria in veratridine-induced neuronal death

Mitochondria play a central role in different cell death pathways (Green and Reed, 1998; Kroemer and Reed, 2000). Apoptosis might involve changes in mitochondrial potential and, in some models, formation of the mitochondrial permeability transitory pore (Bernardi et al., 2001).

First, we studied if veratridine produced any effect on Δ\(ψ_m\) by using the fluorescent dye rhodamine-123. Neuronal cultures responded to veratridine treatment releasing rhodamine-123 in a dose-dependent manner (Fig. 6A). Vera-
the positive cells percentage from at least two different cultures. Data represent mean percentage of cell viability. At least nine coverslips obtained from three different cultures were used for each condition. ***P<0.001 as compared with vehicle.

To study the mitochondrial role in p53 activation pathway we used the PTP inhibitors cyclosporin A (CsA) and bongkrekic acid. Treatments of neuronal cultures with CsA were added 5 min before veratridine and maintained during the experiment. Neurons were bathed in a 0 Ca\(^{2+}\) medium for 10 min prior to veratridine and kept in that medium for 1 additional hour. MnTBAP (10 nM) was present 12 h before veratridine and maintained during the experiment. Neurons were exposed to veratridine (30 \(\mu\)M; 30 min) and 7 h later, the number of immunoreactive positive neurons was determined by counting, at least, 150 neurons in 10 different fields for each coverslip. Data represent mean±S.E.M. of the positive cells percentage from at least two different cultures.

Veratridine-induced collapse in \(\Delta \varphi \) m required Ca\(^{2+}\) influx, since veratridine (30 \(\mu\)M) was ineffective to dissipate \(\Delta \varphi \) m in the absence of extracellular Ca\(^{2+}\) (Fig. 6B).

To address this issue, we measured \(O_2^-\) production by using the rate of conversion of dihydroethidine to ethidium. Hippocampal neurons have a basal \(O_2^-\) production rate of 0.78±0.12 A.F.U. min\(^{-1}\) (n=56; Fig. 7A). Veratridine produced a dose de-
To determine whether the neuroprotective effect of blocking p53 expression was specific on veratridine-induced toxicity, we analyzed the effect of this p53 antisense oligonucleotide (p53as, 1 µM) was added to the cultures 1 h before veratridine exposure. Veratridine-elicited p53 immunoreactivity was abolished by 1 µM p53as treatment and not by a mismatch oligonucleotide (Fig. 4). Indeed, p53 antisense, but not the mismatch, oligonucleotide pre-treatment significantly prevented veratridine-induced neuronal loss (Fig. 8).

To determine whether the neuroprotective effect of blocking p53 expression was specific on veratridine-induced toxicity, we analyzed the effect of this p53as on neuron viability following three insults: oxygen and glucose deprivation, NMDA and the protein kinase C inhibitor staurosporine. As shown in Fig. 8, p53as protected neuronal cultures only in oxygen and glucose deprivation (20 min) and NMDA (100 µM, 20 min) insults but was unable to protect neuronal cultures against the decrease in neuronal cell viability produced by staurosporine (Fig. 8). Oligonucleotides alone did not modify either veratridine-induced
 breaks by ROS (Lof and Poulsen, 2000) might mediate activated endonucleases and induction of DNA strand linking an increase in p53 levels to apoptosis is not known. al., 1997b), excitotoxic response to glutamate (Uberti et al., different noxious stimuli like ionising radiation (Jordan et pressor p53 immunoreactivity in hippocampal neurons. Inviability was analyzed 24 h later. Data represent mean/H11006 *** 30 min), oxygen and glucose deprivation (OGD; 20 min) or NMDA (100 μM, 20 min) exposures or staurosporine (100 nM) addition. Cell viability was analyzed 24 h later. Data represent mean±S.E.M. of at least nine different coverslips obtained from three different cultures. *** P<0.001 as compared with vehicle.

Ca²⁺ influx or O₂⁻ production (data not shown).

**DISCUSSION**

The data presented in the paper suggest that p53 plays a role in the mechanisms involved in the cytotoxic actions of veratridine on rat hippocampal neurons. Veratridine triggers an increase in [Ca²⁺], that yields to a collapse in Δφ m and an increase in the rate of neuronal O₂⁻ production. Veratridine-induced death seems to require activation of a death program, since the transcription factor p53 is activated during the process and its blockade, using an antisense oligonucleotide, significantly prevents veratridine-induced toxicity. Herein, we present evidence that Ca²⁺ and ROS mediate veratridine-induced p53 activation, and how mitochondria could participate in this pathway in rat hippocampal neurons.

In good agreement with activation of a cell death program, veratridine (30 μM, 30 min) increases tumor suppressor p53 immunoreactivity in hippocampal neurons. Increases in p53 protein levels have been found following different noxious stimuli like ionising radiation (Jordan et al., 1997b), excitotoxic response to glutamate (Uberti et al., 1998) and ischemia (Chung et al., 2002). The pathway linking an increase in p53 levels to apoptosis is not known. Both DNA fragmentation induced by activation of Ca²⁺-activated endonucleases and induction of DNA strand breaks by ROS (Lof and Poulsen, 2000) might mediate p53 activation by veratridine.

An increase in [Ca²⁺], might be a common mechanism mediating neurotoxicity induced by different insults including cerebral hypoxia, epilepsy and neurodegenerative diseases. Veratridine depolarizes hippocampal neurons in a dose-dependent fashion achieving, together with action potential firing, membrane potential levels that might open voltage-dependent Ca²⁺ channels and produce [Ca²⁺], elevations. So, it is tempting to suggest that this Ca²⁺ influx is mainly related to activation of voltage-dependent Ca²⁺ channels, since several Ca²⁺ antagonists show some neuroprotective actions on veratridine-induced neurotoxicity on both hippocampal neurons and granular cerebellar cells (Pauwels et al., 1991). Consistently, extracellular Ca²⁺ withdrawn or pretreatment with the cell-permeant Ca²⁺ chelator BAPTA-AM, render neurons resistant to veratridine-induced toxicity. This neuroprotective action of Ca²⁺ chelators is similar to that described in other models of neurodegeneration (Prehn et al., 1996, 1997). Indeed, veratridine-induced neurotoxicity seems to be independent of glutamate receptor activation since the NMDA receptor antagonist, MK-801, was unable to prevent veratridine-induced death and has no effect on veratridine-induced [Ca²⁺] increase.

Mitochondria are the most important intracellular organelles removing Ca²⁺ from the cytosol and the main source of ROS into the neurons (Bindokas et al., 1996). Data presented in Fig. 7B showing that CsA prevents veratridine-induced O₂⁻ generation strongly suggest that mitochondria are the source of veratridine-induced increase in ROS. Supraphysiological increases in [Ca²⁺], might cause PTP opening (Galindo et al., 2003; Reynolds and Hastings, 1995; Schinder et al., 1996). Although the role of mitochondrial swelling has not been characterized, our previous studies (Galindo et al., 2003), using isolated brain mitochondria, showed that Ca²⁺, but not O₂⁻ anions, was responsible for Δφ m collapse. Additionally, using chromaffin cells (Jordan et al., 2002), we showed that veratridine-induced O₂⁻ causes PTP formation and release of cytochrome c with the corresponding caspase cascade activation. These mechanisms seem to be activated in hippocampal neurons as we found that veratridine-induced neuronal death was blocked by caspase inhibitors (Jordan et al., unpublished data) and we observed one of plausible consequence of PTP formation: Δφ m collapse. Veratridine caused a dose-dependent collapse of Δφ m that is dependent upon the presence of extracellular Ca²⁺. A role for mitochondria in veratridine-induced hippocampal neuronal death receives further support by the neuroprotection observed in presence of the well-known PTP blockers cyclosporin A and bongkrekic acid. Furthermore, the present data suggest that PTP formation is mediating p53 induction, since cyclosporin A and bongkrekic acid markedly decreased p53-like immunoreactivity observed after veratridine treatment.

On the other hand, Δφ m collapse might result in ROS production (Reynolds and Hastings, 1995) that might contribute to different signaling pathways leading to neuronal death (Greenlund et al., 1995; Jordan et al., 1995). Veratridine induces a dose-dependent increase in the rate of O₂⁻ production in neuronal cultures that requires Ca²⁺ influx. Although other free radicals, like H₂O₂, may also lead to p53 activation (Niwa et al., 2002), our data suggest that O₂⁻ plays a key role in p53-activation response. In agreement with this, striatal p53 DNA-binding induced by methamphetamine injections was markedly attenuated in Cu, Zn-superoxide dismutase transgenic mice (Asanuma
et al., 2002), while treatments with NMDA or dopamine 1 receptor antagonists did not affect p53 induction (Asanuma et al., 2002). The mechanism of p53 activation by ROS is unknown. ROS have previously been shown to directly activate kinases or inactivate phosphatases that target p53 (Xu et al., 2000). The ability of the PTP blockers to inhibit O$_2^-$ production, to prevent p53 immunoreactivity and to protect neurons from veratridine-induced toxicity, suggests that veratridine might induce a vicious cycle involving perturbed [Ca$^{2+}$]i homeostasis, PTP opening, DNA damage and p53 increase.

Our results support the idea that p53 may act as a mediator in neuronal apoptotic death, corroborating the hypothesis that p53 is active during insults to the brain such as those occurring during ischemia or treatment with certain kinds of genotoxic drugs (Enokido et al., 1996; Wood and Youle, 1995). The ability of antisense oligonucleotides to block p53-mediated cell death, as demonstrated in this and other studies (Eizenberg et al., 1996; Uberti et al., 1998), is also consistent with a role for this protein in some forms of neuronal apoptosis, including excitotoxic death (Lakkaraju et al., 2001). It should be noted, however, that p53 antisense did not protect against staurosporine cell death (Strasser et al., 1994). In a similar way, apoptosis of mouse neurons after growth factor withdrawal or culture in low-K$^+$ medium likely is not related to p53 (Enokido et al., 1996; Davies and Rosenthal, 1994). Furthermore, apoptosis during the development of cerebellar granule cells occurs in p53-deficient mice at normal levels, even though these neurons exhibit increased resistance to the effects of x-irradiation and genotoxic drugs such as methyldoxomethanol (Wood and Youle, 1995).

In summary, hippocampal cell death induced by veratridine is, at least in part, p53-dependent. Therefore, p53 may form an important link between toxic stimuli of varying types and neuronal death. This hypothesis is supported by the finding that hippocampal neurons treated with p53 antisense displayed partial resistance toward veratridine-induced apoptosis. Further studies will be necessary to elucidate subsequent molecular events and cellular dysfunction linked with such veratridine-induced p53 activation.

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