Blockade of Phencyclidine-Induced Cortical Apoptosis and Deficits in Prepulse Inhibition by M40403, a Superoxide Dismutase Mimetic

CHENG WANG, JUSTIN MCINNIS, J. BRETT WEST, JINFENG BAO, NOELLE ANASTASIO, JON A. GUIDRY, YANPING YE, DANIELA SALVEMINI, and KENNETH M. JOHNSON

Departments of Pharmacology and Toxicology (C.W., J.M., J.B.W., J.B., N.A., J.A.G., Y.Y., K.M.J.) and Psychiatry and Behavioral Sciences (C.W., K.M.J.), University of Texas Medical Branch, Galveston, Texas; and MetaPhore Pharmaceuticals (D.S.), St. Louis, Missouri

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ABSTRACT

Repetitive administration of phencyclidine (PCP) in the perinatal period results in cortical apoptosis and a long-lasting deficit in sensorimotor gating. Because these changes are olanzapine-sensitive, we have suggested that the effects of perinatal PCP could be used to model certain aspects of schizophrenia. Studies of PCP and N-methyl-D-aspartate-induced cell death suggested that superoxide could play a role in the pathway leading to death after PCP administration. The purpose of the current study was to determine whether the in vivo administration of M40403, a superoxide dismutase mimetic, could prevent PCP-induced cortical apoptosis and/or deficits in prepulse inhibition. Perinatal rat pups were administered 10 mg/kg PCP on postnatal (PN) days 7, 9, and 11 with or without treatment with 10 mg/kg M40403. Pups were either killed on PN 12 for analysis of various apoptotic markers or they were assessed for prepulse inhibition on PN 24 to 26. Treatment with M40403 2 and 24 h after each PCP treatment prevented PCP-induced increases in two measures of apoptosis in the dorsolateral frontal cortex and in the olfactory cortex. PCP-induced proapoptotic changes in Bax and Bcl-XL were also prevented by M40403 treatment. This regimen did not prevent the deficit in prepulse inhibition caused by PCP treatment, but when the treatment regimen was extended through PN 23, M40403 completely prevented the PCP-induced deficit in prepulse inhibition. These data suggest that perinatal PCP treatment leads to long-lasting changes in the pathway(s), leading to cell death and behavioral deficits, and that the superoxide radical plays a critical role in the underlying mechanism.

Phencyclidine (PCP) administration in humans mimics many of the signs and symptoms of schizophrenia (Luby et al., 1959; Javitt and Zukin, 1991). Long-term use of PCP is associated with reduced blood flow and glucose utilization (Hertzzmann et al., 1990; Cosgrove and Newell, 1991), which is similar to what is observed in schizophrenic patients (Weinberger et al., 1986; Lahti et al., 1995).

The neurotoxic effects of NMDA antagonists such as PCP and MK-801 (Olney et al., 1989, 1991) have been related to the structural deficits found in schizophrenia (Goldstein et al., 1999; Pearlson and Marsh, 1999). More recently, it has been demonstrated that mice having only 5% of the normal levels of the obligatory NR1 subunit of the NMDA receptor display a behavioral phenotype characterized by increased locomotor activity and deficits in social and sexual interactions (Mohn et al., 1999). Although schizophrenia is known not to be associated with dramatic loss of NMDA receptors, the observation that these behavioral deficits could be ameliorated with antipsychotic drugs supports the general hypothesis that “NMDAergic” hypofunction may underlie certain features of schizophrenia (Olney and Farber, 1995). It is possible that the neuroanatomical deficits associated with schizophrenia combine to produce a state that closely resembles hypofunction of NMDA-mediated neurotransmission.

Microscopic examination of post-mortem brain tissue has led other investigators to postulate that the loss of neurons and/or a developmental deficit, particularly in subregions of the frontal cortex, underlies many core symptoms of schizophrenia (Benes et al., 1991, 1995; Goldman-Rakic and Selemon, 1997; Lewis, 1997; Goldman-Rakic, 1999). It has been suggested that in at least some cases of schizophrenia, the primary pathological insult may occur in the pre- or perinatal period (Benes et al., 1991; Murray et al., 1992;
Pilowski et al., 1993; Goldman-Rakic and Selemon, 1997). Apoptosis, or programmed cell death, could play a role in either neuronal loss or in aberrant neural development (Weinberger, 1987). Although such a multifaceted disease will be difficult to model completely, it has been demonstrated that repetitive PCP use results in persistent symptoms of schizophrenia (Carlin et al., 1979; Krystal et al., 1984; Cosgrove and Newell, 1991). Repetitive PCP administration in adult rats has been reported to produce behavioral, cellular, and biochemical deficits sensitive to antipsychotics (Jentsch et al., 1997; Johnson et al., 1998).

It seems unlikely that the behavioral and underlying neuronal plasticity observed in adult animals could completely mimic the changes in the developing brain initiated by a perinatal insult. Therefore, we recently determined the effect of perinatal PCP treatment on cortical NMDA receptors, cell death, and various elements of a proposed cell death pathway as well as on prepulse inhibition of acoustic startle (Wang et al., 2001). All of the measures altered by PCP were prevented by olanzapine pretreatment, thereby suggesting the validity of this paradigm as a model of schizophrenia. We have proposed that because perinatal PCP treatment leads to a robust increase in NR1 mRNA and protein, the mechanisms leading to cell death in this model are probably similar to those seen in vitro after the addition of toxic concentrations of NMDA to forebrain cultures (McInnis et al., 2002). In that study, we reported that NMDA-induced cell death was critically dependent on the formation of superoxide anion (O2•). Therefore, in the current study we proposed that changes in prepulse inhibition as well as markers of cortical apoptosis caused by repetitive PCP administration in vivo could be prevented by M40403, a nonpeptidyl superoxide dismutase mimetic.

Materials and Methods

Experimental Design. Timed pregnant female Sprague-Dawley rats were obtained on day 14 of pregnancy from Charles River Laboratories, Inc. (Wilmington, MA). They were housed individually with a regular 12-h light/dark cycle (lights on at 7:00 AM, off at 7:00 PM) and food and water available ad libitum. Within 12 h of parturition, the pups from five dams were combined and then randomly cross-fostered to one of five lactating dams. Each litter consisted of six to eight pups. On postnatal day (PN) 7, 9, and 11, the pups were treated with PCP or saline followed by M40403 or saline post-treatment as described below.

For biochemical and histochemical studies, pups were killed on PN 12, 24 h after the last PCP administration. Various brain regions were then processed for TUNEL assays, fragmented DNA detection by ELISA, and/or Western blot analyses. For behavioral studies, the pups were weaned at 21 days of age and on PN 25 to 26, the rats were tested for baseline prepulse inhibition of acoustic startle.

Drugs and Treatments Paradigm. In the biochemical and histochemical experiments, rat pups were grouped into four (terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) and ELISA) or five (Western blot analysis) treatment conditions. The pups were treated with either saline or 10 mg/kg s.c. PCP followed by administration of 10 mg/kg s.c. M40403 (2 and 24 h after each PCP injection). Thus, the five treatment groups were saline/saline (control), PCP/saline, saline/M40403, PCP/M40403, and PCP/M40404 (an inactive analog of M40403, at 10 mg/kg s.c.). The latter group was used only in Western blot experiments.

In the behavioral studies, the pups were treated with either saline or PCP followed by administration of M40403. The five treatment groups were saline/saline (control), PCP/saline, saline/M40403, PCP/M40403 (short-term; 2 and 24 h after each PCP injection), and PCP/M40403 (long-term). The pups in the long-term group were treated with M40403, 2 and 24 h after each PCP injection, and then once per day on PN 13 to 24 before testing in the prepulse inhibition paradigm on PN 25 to 26. PCP was acquired from the National Institute on Drug Abuse (Rockville, MD). M40403, a nonpeptidyl superoxide mimetic, and its inactive control (M40404) were synthesized at MetaPhore Pharmaceuticals as described previously (Salvemini et al., 1999). PCP was dissolved in 0.9% NaCl. M40403 and M40404 were dissolved in 26 mM sodium bicarbonate, pH 8, and injected s.c.

Assessment of Neurotoxicity. Nucleosomal DNA fragmentation is characteristic of apoptotic nuclei (Gavrieli et al., 1992; Rabacchi et al., 1994). Condensed or fragmented DNA was assayed using the TUNEL essentially as described previously (Johnson et al., 1998; Wang et al., 2001). Deoxynucleotidyl transferase (TdT), a template-independent polymerase, was used to incorporate biotinylated nucleotides at sites of DNA breaks. The signal was amplified by avidin-biotin peroxidase, enabling conventional histochemical identification by light microscopy. In brief, the brain sections were treated with proteinase K to dissociate proteins from DNA and the sections were then washed in phosphate-buffered saline (PBS). Endogenous peroxidase was inactivated by covering the sections with H2O2. The sections were rinsed with PBS and covered with 2% bovine serum albumin for 10 min at room temperature. Sections were then rinsed in PBS solution and covered with 1:50 dilution of avidin-biotin complex (ABC; Vector Laboratories, Burlingame, CA), incubated for 30 min at 37°C, and immersed in 0.05% 3,3′-diaminobenzidine (Sigma-Aldrich, St. Louis, MO). For negative controls, TdT was omitted from the reaction mixture. As a positive control, the brain sections were treated with 1 N HCl for 20 min before the terminal transferase.

Photographs of the TUNEL-stained sections from representative regions of the dorsolateral frontal cortex, striatum-nucleus accumbens, hippocampus, and cerebellum were made and the number of TUNEL-positive cells in the frontal cortex were evaluated by three independent observers, two of whom were blind to the treatment. The mean counts from the three observers were used to calculate the mean and S.E.M. from five animals in the four treatment groups. The other regions contained very few TUNEL-positive cells in any treatment group and only the striatum was quantitated.

The presence of fragmented DNA associated with nucleosomal histones is thought to be a more specific marker for apoptosis than the TUNEL assay. Therefore, this was assessed in perinatal brains with the use of a specific two-site ELISA using an anti-histone primary antibody and a secondary anti-DNA antibody according to the manufacturer’s instructions (Roche Applied Science). Briefly, the dorsal frontal cortex was dissected and the sections were homogenized in 3 ml of lysis buffer with approximately 15 strokes of a 1-ml manual Wheaton Tenbroeck tissue grinder and incubated for 30 min at room temperature. After centrifugation, the supernatants (cytosol containing low-molecular mass, fragmented DNA) were diluted 1:2 (v/v) with lysis buffer. Then, 20 μl from each sample was transferred to a plate reader well precoated with anti-histone antibody, and 80 μl of immunoreagent mix, including the secondary antibody, was added. After incubation and washes, the wells were treated with the chromogen substrate, and the intensity of the color that developed was assayed at 405/490 nm.

Western Blot Analysis. The dorsal frontal cortex was dissected and the sections were homogenized in lysis buffer (radioimmunoprecipitation assay buffer) with approximately 15 strokes in a 1-ml manual Wheaton Tenbroeck tissue grinder. Radioimmunoprecipita-
tion assay buffer consisted of 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS solution. Just before using, 1 mM phenylmethylsulfonyl fluoride and 450 U/ml aprotinin were added to the lysis buffer. After centrifugation, samples were measured for protein concentration with BCA protein reagent (Pierce Chemical, Rockford, IL). Equal amounts of total protein (10 μg) were loaded on each lane and run on SDS-polyacrylamide gels with a Tris-glycine running buffer system and then transferred to a polyvinylidene difluoride membrane (0.2 μm) in a Mini Electrotransfer Unit (Bio-Rad, Hercules, CA). The blots were probed with an anti-Bcl-xL (1:2000, polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA) antibody and anti-Bax (1:1000, polyclonal; Santa Cruz Biotechnology) antibody. Immunoblot analysis was performed with horseradish peroxidase-conjugated anti-rabbit IgG using the enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). The Bcl-xL/Bax ratio was analyzed by an automated image analysis system (Alpha Innotech Corporation, San Leandro, CA).

**Prepulse Inhibition.** Measurement of prepulse inhibition of acoustic startle was accomplished according to minor modifications (Wang et al., 2001) of a procedure outlined previously (Pietraszek and Ossowska, 1998). Testing was performed between 9:00 AM and 3:00 PM using two startle chambers (SR-LAB; San Diego Instruments, San Diego, CA) that were placed in a sound-attenuated room with a 60-dB ambient noise level. After acclimation for 5 min, each rat was exposed to two stimulus types: a 120-dB, 40-ms pulse or a pulse preceded 100 ms earlier by a 20-ms noise burst of 68 dB (prepulse), with a variable intertrial interval for a total of 40 trials (20 prepulse trials and 20 pulse alone trials). In preliminary experiments, we used a paradigm that also measured the response to no stimulus (baseline) and to prepulse alone. PCP treatment had no effect on these measures and thus, these measurements were not included in our routine experimental paradigm. The percentage prepulse inhibition was calculated as the percentage of inhibition of the startle amplitude evoked by the pulse alone: [pulse − (prepulse + pulse)/pulse] × 100.

**Results**

As we have observed previously (Wang et al., 2001), treatment of rat pups on PN 7, 9, and 11 with 10 mg/kg PCP caused an increase in TUNEL-positive staining of cells in the cortex on PN 12. In saline-treated pups, the only TUNEL-positive cells were in or just below the meningeal layer (Fig. 1A), a normal characteristic of this rapidly turning-over cell type. After PCP, an increased density of staining was most evident in the piriform cortex, olfactory tubercles, and dorsolateral region of the frontal cortex (Fig. 1C). Within the dorsolateral frontal cortex, there is evidence of enhanced TUNEL staining in layers I to III. This diminished significantly in deeper layers (data not shown). M40403, the superoxide dismutase mimic, had no effect alone (Fig. 1B) but was able to completely blunt the effect of PCP (Fig. 1D).

These observations were validated by quantitative analysis of the TUNEL staining (Fig. 2, top), which showed that PCP increased the number of TUNEL-positive cells in the dorsolateral frontal cortex by more than 4-fold. Because there were very few TUNEL-positive cells in the striatum, the entire left or right (randomly selected) striatum was counted. Striatata from control rats contained 7.5 ± 0.89 TUNEL-positive cells per side. PCP treatment did not alter this significantly (9.0 ± 1.9). Animals treated with M40403 alone and M40403 plus PCP had 6.3 ± 1.3 and 5.7 ± 1.0 TUNEL-positive cells, respectively, per side. These treatments were not significantly different than control (F = 1.18, df = 3, p = 0.34). Thus, PCP treatment caused a regionally selective neurotoxicity after this dosage regimen.

Because the TUNEL stain is known not to be absolutely specific for apoptosis, we also assessed apoptosis in a completely independent experiment by measuring the amount of fragmented DNA associated with histone proteins in a double antibody ELISA (Fig. 2, bottom). This measurement indicates that PCP treatment approximately doubles the concentration of DNA-associated histone and that this measure of PCP-induced apoptosis is also completely blunted by M40403 treatment.

We have previously demonstrated in this model that PCP treatment both increases Bax and decreases Bcl-xL in the frontal cortex (Wang et al., 2001). Furthermore, we have demonstrated that the increase in Bax after NMDA treatment of dissociated forebrain cultures occurs temporally in a manner corresponding to the development of apoptotic markers (McInnis et al., 2002). Therefore, we determined the effect of M40403 on Bax and Bcl-xL after PCP treatment. Figure 3, top, shows a representative Western blot from these experiments. Again, PCP causes an increase in Bax and a decrease in Bcl-xL. These effects were completely normalized by treatment with M40403. An inactive analog of M40403 is M40404 and it was used to control for nonspecific effects of this class of molecules. As can be seen, it had no effect on the changes caused by PCP treatment. After estimation of band densities by densitometry, the ratio of Bcl-xL/Bax was quantitated and these data are shown in Fig. 3, bottom. The approximate 80% decrease in Bcl-xL/Bax caused by PCP treatment is similar to the 60% decrease we have previously observed in this model (Wang et al., 2001).

Finally, we tested the effect of M40403 treatment on the decrease in prepulse inhibition caused by PCP treatment (Fig. 4). First, the approximate 60% prepulse inhibition observed in control pups is similar to what we have observed previously using this paradigm (Wang et al., 2001). However, the effect of PCP treatment (80% decrement in prepulse inhibition) is larger than the 40 to 55% decrements previously observed.

Treatment with M40403 2 and 24 h after each of the PCP treatments did not have a protective effect on prepulse inhi-
Discussion

There are two major findings of this study. First, we confirmed our original observations that perinatal PCP exposure results in long-lasting deficits in a behavioral measure of sensorimotor gating and that measure is associated with evidence of enhanced cortical apoptosis, including increased DNA fragmentation and TUNEL staining as well as a reduction in the Bcl-XL/Bax ratio (Wang et al., 2001). Second, the ability of M40403 to prevent both the biochemical and behavioral sequelae of perinatal PCP administration strengthens the causal relationship between cortical cell death and deficits in prepulse inhibition and in doing so suggests that this treatment protocol may result in behavioral and biochemical alterations that may model important aspects of the development of schizophrenia. Finally, the effectiveness of M40403 in this paradigm suggests a potential novel therapeutic approach for those individuals at risk for schizophrenia and perhaps other conditions such as stroke in which overactivation of NMDA receptors may play a role.

Reduced Prepulse Inhibition with Perinatal PCP. For perinatal treatment with PCP to have some face validity as a model of schizophrenia, it is necessary for the resulting behaviors to have some relevance to the disease. Schizophrenic patients are known to have difficulty in filtering information from their surroundings and have deficits in sensorimotor gating as measured by prepulse inhibition (Braff et al., 1992). Rats and healthy humans also show deficits in prepulse inhibition after treatment with NMDA antagonists (Mansbach and Geyer, 1989; Bakshi et al., 1994; Karper et al., 1994). Clozapine and olanzapine have been shown to prevent PCP-induced prepulse inhibition deficits (Bakshi et al., 1994; Bakshi and Geyer, 1995), but haloperidol does not (Keith et al., 1991). This profile suggests that prepulse inhibition deficits may be a reasonable model of the negative symptoms of schizophrenia. With this in mind, we previously postulated that perinatal PCP treatment should produce a deficit in baseline prepulse inhibition that could be prevented by olanzapine (Wang et al., 2001). The ability of olanzapine pretreatment to prevent the development of this deficit in prepulse inhibition further supports the hypothesis that perinatal PCP may also model the negative symptoms of schizophrenia. This notion was further supported by another experiment showing that olanzapine not only could prevent the deficit in prepulse inhibition when administered just before PCP but also was able to prevent the effects of chronic PCP when administered for 12 days beginning 1 day after PCP administration (Wang et al., 2001).

PCP-Induced Neurotoxicity and Underlying Mechanisms. There are a variety of mechanisms that could underlie the neurotoxic effect of PCP. At the cellular level, one possibility is that neurons that are deprived of “NMDAergic” input for a sustained period may undergo degeneration (Ikonomidou et al., 1999). Another possibility is that NMDA antagonists increase glutamate release (Moghaddam et al., 1997) and if this mechanism were still operative after chronic PCP administration as suggested by others (Arvanov and Wang, 1998), this could contribute to the observed neurotoxicity and associated behavioral effects. It is also possible that normal glutamatergic transmission during the period when PCP concentration is low is actually enhanced because of the up-regulation of the NR1 receptor subunit (Wang et al.,...
However, NMDA receptor up-regulation alone is not sufficient to cause degeneration because we have observed in previous studies that this receptor subunit is up-regulated in regions such as the caudate that do not show signs of degeneration (Johnson et al., 1998; Hanania et al., 1999; Wang et al., 1999, 2001). Thus, in addition to increased function of NMDA receptors, other factors must also be involved. These factors could involve the stage of synaptogenesis or composition of the NMDA receptor subunits in different brain regions as suggested by others (Ikonomidou et al., 1999). It is also possible that death of glutamatergic projections neurons is actually neuroprotective to the afferent region, e.g., death of corticostriatal glutamatergic neurons could protect the striatum from glutamate-dependent cell death. Regional selectivity could also be provided by differences in the regulation and supply of various growth factors, antioxidant homeostasis, DNA repair mechanisms, and/or caspase activation cascades.

**Subcellular Mechanisms of PCP-Induced Apoptosis.** We have previously observed that NMDA application in vitro induces death of forebrain neurons by both necrosis and apoptosis, with necrosis preceding apoptosis (McInnis et al., 2002). Apoptosis was accompanied by an increase in nuclear factor-κB (NF-κB) nuclear translocation and an increase in the proapoptotic protein Bax and a decrease in the antiapoptotic protein Bcl-XL (McInnis et al., 2002). Because cultures pretreated with PCP were more sensitive to this effect of NMDA (Wang et al., 2000a), we reasoned that similar mechanisms might play a role in neurodegeneration of the frontal cortex of PCP-treated rat pups. This hypothesis was confirmed previously (Wang et al., 2001) and again in the present study. The mechanism by which NMDA up-regulates Bax is not completely understood, but the prevention of this effect in vitro by the addition of catalase and superoxide dismutase (Wang et al., 2000a) or M40403 (McInnis et al., 2002) suggests the possible involvement of reactive oxygen species, and superoxide in particular. The link between the formation of superoxide and regulation of either Bax or Bcl-XL in this system is also unknown. The tumor suppressor p53 and the transcription factor NF-κB are known to be sensitive to the redox state of the cell and both are induced by glutamate in primary cultures (Grilli et al., 1996; Uberti et al., 1999). As such, they are possible candidates for mediating this linkage. The proapoptotic tumor suppressor protein p53 is known to up-regulate Bax in several settings (Chao and Korsmeyer, 1998), which has been implicated in several p53-dependent models of apoptotic cell death (Xiang et al., 1998; Cregan et al., 1999). Our recent study of NMDA-induced neurodegeneration demonstrated that nuclear translocation of NF-κB proteins precedes the increase in Bax and DNA fragmentation and both could be prevented with either M40403 or SN50, a peptide inhibitor of NF-κB protein translocation (McInnis et al., 2002). These data suggested that superoxide anion activates an apoptotic pathway involving NF-κB transcription factors, possibly through activation of IκB kinases that are known to be sensitive to the redox status of the surrounding milieu. Phosphorylation of IκB targets this protein for ubiquination and ultimately releases NF-κB transcription factors, including p50, p52, p65, and c-Rel, which then can activate the trans-
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cription of many genes, including Bax. Whether the scenario described above is operative in vivo after the treatment of rat pups with PCP is uncertain, but the ability of M40403 to prevent PCP-induced increases in Bax and cortical neurodegeneration are strongly supportive of such a mechanism. Furthermore, the correlation between the preventative effects of M40403 on PCP-induced increases of both Bax and TUNEL-positive cells supports the notion that PCP induces an apoptotic cell death as suggested previously (Ikonomidou et al., 1999; Wang et al., 2001).

Summary. We have presented evidence showing that perinatal PCP treatment results in long-lasting alterations in a model of sensorimotor gating that may be related to behavioral changes observed in schizophrenia. Evidence is also presented suggesting that these behavioral disturbances could be related to the apoptotic loss of neurons in the cortex. The loss of cortical glutamatergic neurons that regulate subtotal dopaminergic input to the cortex could account for the ability of this model to mimic the hypoglutamatergic state thought to be critical in schizophrenia (Olney and Farber, 1995). These results also suggest that neurotoxicity in this model could be the consequence of increased superoxide anion production and subsequent effects on the regulation of Bax and Bcl-XL, two proteins known to play a critical role in anion production and subsequent effects on the regulation of this model could be the consequence of increased superoxide production, and subsequent effects on the regulation of Bax and Bcl-XL, two proteins known to play a critical role in p53-induced apoptosis in neurons. Neuropharmacology 271:757–794.


