NMDA Agonists and Antagonists Induce Renal Culture Cell Toxicity

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Abstract: The NMDA receptor (NMDAR) is expressed in the renal proximal tubule. NMDAR agonists and antagonists induce cell toxicity in the central nervous system (CNS). We studied the effect of NMDAR agonists and antagonists on renal cell survival in renal culture cells: proximal tubule-like opossum kidney (OK) and distal-tubule-like madine darby canine kidney cells (MDCK) cells. Low dose glutamate had no effect on cell survival. However, 10 mM glutamate induced a 14-fold increase in cell death compared to control cells. Addition of low or high doses of the NMDAR agonist glycine had no effect on cell toxicity. Exposure of cells to the non-competitive NMDAR blocker MK-801 or the competitive NMDAR antagonist CPP induced a time and dose-dependent increase in cell death and apoptosis. The presence of fetal bovine serum in the pre-incubation media attenuated the toxicity caused by MK-801 and CPP. The deleterious effect of NMDAR antagonists on cell survival was specific for OK cells; these substances had no effect on MDCK cell survival. Finally, pre-treatment of OK cells with the renal cytoprotective glycine completely blunted the affect of MK-801 on renal cell survival. We conclude that excessive stimulation or blockade of the renal NMDAR results in cell death.

Key Words: NMDA receptor, kidney, agonists, antagonists, cytotoxicity.

INTRODUCTION

The N-methyl-D-Aspartate (NMDA) receptor (NMDAR) is linked to a cation channel. The main NMDA subunit is NR1, which is linked to one of the NR2 subunits (NR2A, NR2B, NR2C, or NR2D) [1-3]. The NR1 subunit is essential for channel activity whereas the NR2 subunits confer modulatory properties [4]. Cations transmitted through the NMDAR-linked ion channel include sodium, potassium, and calcium [2]. Activation of the central nervous system (CNS) NMDAR results in ototoxicity [5,6] and hypoxia [1,7,8].

Studies in ours and other laboratories show that the NMDAR is abundantly expressed in the renal proximal tubule [9,10] with moderate distal tubule expression. Increased NMDAR subunit NR1 and NR2C expression correlates with renal damage in a rat model of gentamicin nephrotoxicity [11]. Thus, similar to the CNS NMDAR [1,5-7,8], activation of the receptor mediates renal cell toxicity. However, NMDAR antagonists also mediate CNS *in vitro* and *in vivo* cell toxicity [12-14]. For example, NMDAR antagonists induce cytotoxicity and apoptosis in microglia, a phenomenon that is ameliorated by simultaneous addition of NMDA agonists [14]. Thus, there seems to be a delicate balance by which excessive stimulation or blockade of the NMDAR results in altered cell survival.

There is no published information regarding the effect of NMDAR agonists or antagonists in the kidney. We studied the effect of several potential NMDAR agonists and antagonists on renal cell survival in proximal tubule–like opossum kidney (OK) and distal tubule-like madine darby canine kidney (MDCK) cells.

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METHODS

Cell Culture

Opossum kidney (OK) and Madine Darby Canine Kidney (MDCK) cells from established lines were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were grown in Minimal Essential Medium Eagles (30-2003, ATCC), and grown to 80-90% confluence with addition of 10% fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 μg/ml streptomycin in 5% CO_2-95% O_2 at 37°C.

Assessment of Cytotoxicity

Cell toxicity was assessed by lactate dehydrogenase (LDH) release according to an established protocol. Briefly, toxicity was determined by the *In Vitro* Toxicology Kit, or TOX-7^R (Sigma-Aldrich, Saint Louis, MO, USA). The assay is based upon the reduction of NAD by the action of LDH. The resulting reduced NAD (NADH) is utilized in the stoichiometric conversion of a tetrazolium dye. The night before all experiment cells were incubated for 16 hours with FBS-deplete or replete media. The next day, these solutions were replaced with fresh solutions containing NMDA agonists or antagonists and then incubated for 2 or 24 hours. The cultures were then removed and placed in a laminal flow hood. The plates were then centrifuged at 250 X g for 4 minutes. An aliquot was then transferred to a 96-well plate for LDH release analysis. To the remainder of the cells/media in the plate, 1/10 volume of 0.25% Triton X-100 was added to induce cell lysis. After incubation 5% CO_2-95% O_2 at 37°C for 45 minutes, the plate was removed from the incubator and centrifuged again at 250 X g X 4 minutes. An aliquot was removed for total LDH analysis. An LDH assay mixture was prepared with equal volumes of LDH enzyme, substrate, and dye solutions. One-half volume of the mixture was added to the wells containing an aliquot from the cells. After

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incubation in the dark for 30 minutes, the reaction was stopped by addition of 1/10 volume 1N HCL. LDH was measured spectrophotometrically at a wavelength of 490 nm, with the background subtracted at 690 nm. The results for the cytotoxicity experiments are displayed as LDH release/Total LDH.

Assessment of Apoptosis

Cell apoptosis was assessed by Hoechst 33258 staining of cells, as described previously [15]. After incubation with the substances under study, the cells were initially washed with 1X phosphate buffered saline. Hoechst 33258 stain (Sigma-Aldrich, Saint Louis, MO, USA) was added in 1X PBS at a concentration of 2 μM and the cells were incubated in the dark in 5% CO_2-95% O_2 at 37°C for 45 minutes. After incubation, apoptosis was assessed at 350/460 nm on an ultraviolet (UV) microscope. Apoptotic cells were identified by bright white staining, which was easily visible by 10X magnification. Normal cells were identified by gray staining. The percentage of apoptosis was determined by the number of apoptotic/total cells/high power field (hpf).

Materials

Glycine, glutamate, MK-801 and R-(-)-3-(2-carboxypiperazine-4-yl)-propyl-1-phosphonic acid (D-CPP, or CPP) were purchased from Sigma-Aldrich (Saint Louis, MO, USA).

Statistical Analysis

Differences among more than two data points was assessed by one-way ANOVA with Bonferroni's Multiple Comparison Test. Significance is defined as $P< 0.05$.

RESULTS

Opossum Kidney (OK) Cell Survival After Exposure to NMDA Agonists

OK cells were exposed to glutamate (0.01-10 mM) for 24 hours. As a positive control for cell toxicity we used high-dose gentamicin (27 mg/ml), which induces significant cytotoxicity to renal culture cells [16]. Compared to control cells, there was no effect on cell toxicity in OK cells exposed to 0.01-1 mM glutamate. However, addition of higher concentrations of glutamate induced a dose-dependent increase in cell toxicity. The highest dose of glutamate (10 mM) resulted in a 14-fold increase in cell toxicity compared to control cells ($p<0.0001$). The toxicity due to this concentration of glutamate was significantly greater ($p=0.002$) than that observed with gentamicin (Fig. **1A**).

A separate group of OK cells were exposed to glycine (10 μM-10 mM) for 24 hours. Compared to control cells, there was no effect on cell toxicity by all doses of glycine; gentamicin induced greater toxicity ($p=0.05$) than the control or glycine (Fig. **1B**).

OK Cell Survival After Exposure to the Non-Competitive NMDA Antagonist MK-801

After overnight incubation in FBS-deplete media, the non-competitive NMDAR antagonist MK-801 was added to OK cells for either 2 (Fig. **2A**) or 24 (Fig. **2B**) hours. Exposure of cells for both time periods to doses higher than 0.10 μM MK-801 induced a dose-dependent increase in cell death.

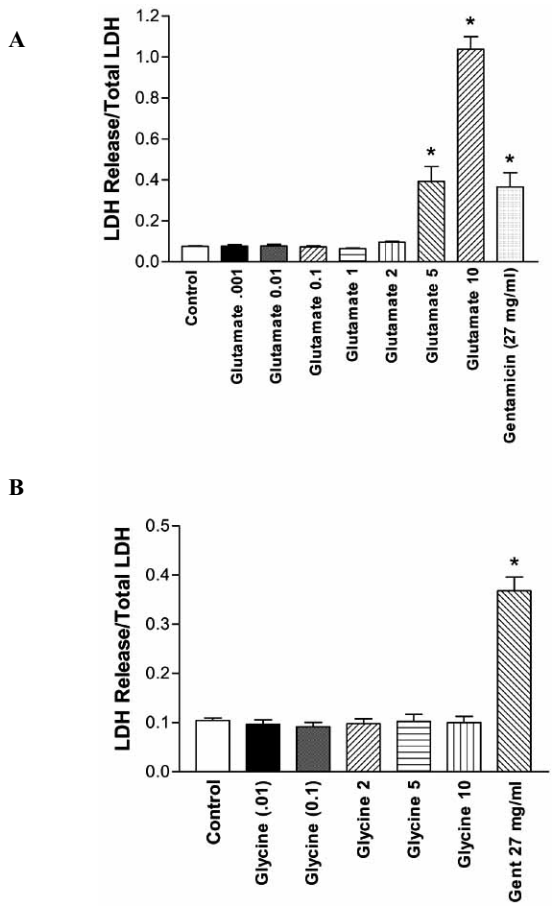

Fig. (1). Cytotoxicity (LDH Release/Total LDH) in OK cells pre-incubated in the absence of FBS and then exposed to 0.001-10 mM glutamate (A) or 0.01-10 mM glycine (B) for 24 hours. There are significant increases (*$p< 0.05$) in cytotoxicity in cells exposed to concentrations 5 and 10 mM glutamate or the positive control high-dose gentamicin (27 mg/ml) versus the control condition. Cells exposed to low or high doses of glycine showed similar levels of cytotoxicity to the control cells; those exposed to high-dose gentamicin (27 mg/ml) exhibited significant toxicity versus either the control or glycine-exposed cells.

Apoptosis in OK Cells Exposed to MK-801

After overnight incubation in FBS-deplete media, OK cells were incubated for either 2 or 24 hours with MK-801. Apoptosis was assessed by Hoechst 33258 staining. The results of a typical experiment are shown (Figs. **3A** and **3B**). 2-hour exposure to MK-801 resulted in a trend towards a dose-dependent increase in apoptosis, but the results were not statistically significant. However, 24-hour exposure of cells to MK-801 resulted in a dose-dependent increase in apoptosis. As a positive control, we measured apoptosis in cells exposed to high-dose gentamicin (27 mg/ml) that induces apoptosis in renal culture cells [17]. Gentamicin induced apoptosis similar to that observed with the higher concentrations of MK-801. The effect of MK-801 on apoptosis in OK cells for either 2 (Fig. **3C**) or 24 (Fig. **3D**) is also displayed

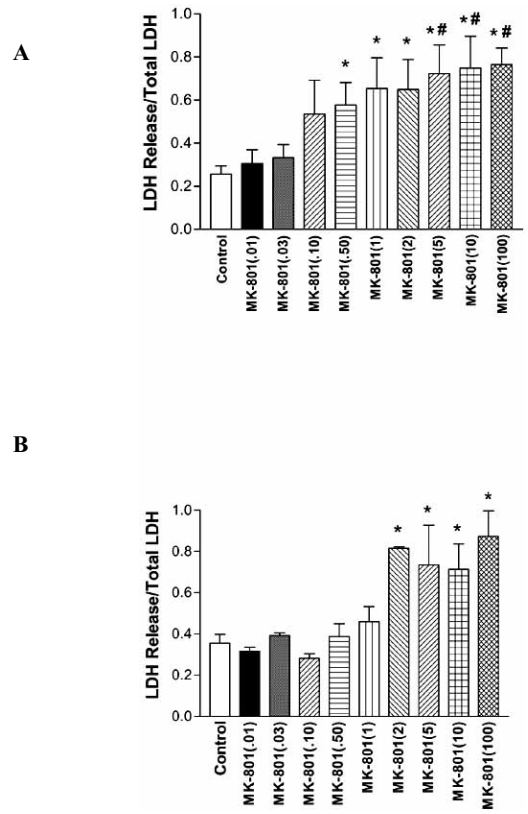

Fig. (2). Cytotoxicity (LDH Release/Total LDH) in OK cells pre-incubated in the absence of FBS and then exposed to 0.01-100 μM MK-801 for 2 (A) or 24 (B) hours. There are significant increases (*p< 0.05) in cytotoxicity in cells exposed to concentrations of MK-801 versus the control condition in all three time periods studied. Cells exposed to higher concentrations (at least 5 μM) of MK-801 for either 2 or 24 hours also exhibited significant differences (# p< 0.05) in toxicity compared to lower concentrations of MK-801.

in pictorial form, confirming a dose-dependent effect of MK-801 on rates of apoptosis.

OK Cell Survival After Exposure to the Competitive NMDA Antagonist CPP

After overnight incubation in FBS-deplete media, the competitive NMDAR antagonist CPP was added to OK cells for either 2 (Fig. **4A**) or 24 (Fig. **4B**) hours. Exposure of cells for both time periods to doses higher than 0.10 μM induced a dose-dependent increase in cell death.

Apoptosis in OK Cells Exposed to CPP

2-hour exposure of cells to CPP resulted in a trend towards increased apoptosis with higher doses of CPP, but the results are not statistically significant (Fig. **5A**). However, 24-hour exposure to CPP resulted in significant and dose-dependent apoptosis (Fig. **5B**). The degree of apoptosis induced by the positive control gentamicin was similar to that observed with the highest concentrations of CPP.

Assessment of Protective Effect of FBS on NMDA Antagonist Induced Cell Toxicity

FBS attenuates cell toxicity induced by various agents [18-20]. Hence, we sought to study if pre-incubation of OK cells with FBS may offer some protection against NMDAR-antagonist induced cell death induced by NMDAR antagonists. The overall rate of cell toxicity was lower in cells exposed to FBS-replete media. Indeed, only maximal doses of MK-801 (Fig. **6A**) and CPP (Fig. **6B**) induced cytotoxicity. These studies show that FBS does offer some protection of renal cells from NMDAR antagonist-induced cell death.

Madine Darby Canine Kidney (MDCK) Survival After Exposure to NMDA Antagonists

The NMDA receptor is also expressed in MDCK cells [10]. Hence, we studied the effect of 24-hour exposure of the NMDAR antagonist MK-801 on MDCK cell survival. These results show that either in the absence or presence of pre-incubation with FBS, cell survival was unaffected by MK-801 (Fig. **7**).

Protective Effect of Glycine on NMDA Antagonist-Induced OK Cell Cytotoxicity

Glycine alone had no effect on renal cell survival (Fig. **1B**). However, pre-treatment of cells with glycine prior to exposure to the nephrotoxicants cisplatin [21], heat stress [22], or hypoxia attenuates cell death [23]. Based on these studies, we speculated that pre-treatment of OK cells with glycine may protect the cells against NMDAR antagonist-induced cytotoxicity. Our results show that pre-treatment of OK cells with low (100 μM), moderate (2 mM), or high-dose (10 mM) glycine offers complete protection against MK-801-induced cytotoxicity (Fig. **8**).

DISCUSSION

The NMDA receptor (NMDAR) plays an important role in cell survival in the central nervous system (CNS), as indicated by its role in excitatory pathways. However, excessive activation of the CNS NMDAR results in cell toxicity and plays a role in Alzheimer's disease, hypoxia, alcoholism, and drug-induced ototoxicity [5,6,8,24-33]. While non-competitive (e.g. MK-801) or competitive (CPP) antagonists ameliorate cell damage in some of these aforementioned conditions, exposure of cells to high doses of these antagonists may also cause cell damage [12-14]. The mechanism underlying antagonist-induced cell toxicity is not clear.

We studied the effect of two major CNS NMDAR agonists on renal cell survival. High dose glutamate induced significant toxicity, even greater than that exhibited by gentamicin. In contrast, low, moderate, or high-dose glycine had no deleterious effect on renal cell survival. We also explored the effect of two NMDAR antagonists on cells survival. Our results show that exposure of OK cells to a non-competitive (MK-801) and competitive (CPP) antagonist causes a dose-dependent increase in cytotoxicity and apoptosis. "*To our surprise, apoptosis occurred in renal culture cells exposed to both a non-competitive and competitive antagonist. This effect at first seems counterintuitive however, the effects of both MK-801 and CPP were dose-dependent, with significant cell death only seen with high doses. This result is similar to those observed by Marcus et al.* [34], *in which both non-competitive and competitive NMDA receptor antagonists evoked dopamine output in limbic cortical regions that were dose dependent. In summary, the ability of both non-competitive and competitive antagonists to induce an effect in a single cell type seems to be dose-dependent. The density*

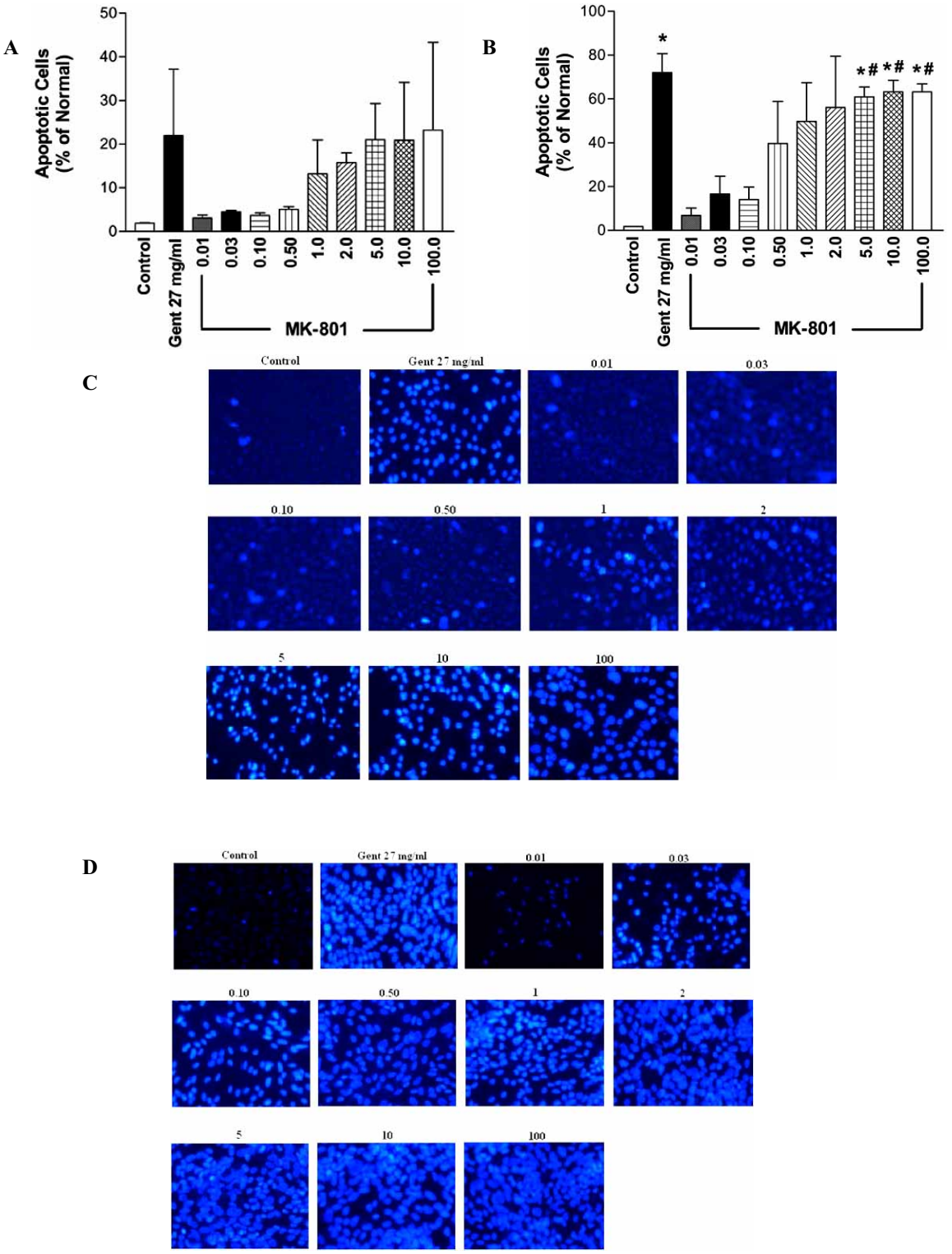

Fig. (3). Apoptosis in OK cells exposed to 0.01-100 μM MK-801. MK-801 was applied to the cells for either 2 hours (**A**) or 24 hours (**B**), and then Hoechst stain 33258 was added to the cells. Apoptotic cells stain white and are easily visible; normal cells are identified by gray staining, and on this reproduction are not easily visible. There is a time and dose-dependent increase in apoptosis in the cells. As a positive control, the cells were exposed to high-dose gentamicin (27 mg/ml). The effect of MK-801 on apoptosis in OK cells for either 2 (Figure IIIC) or 24 (Figure IIID) is also displayed in pictorial form. The numbers above each frame represent μM MK-801 added. Apoptotic cells can be identified by bright blue-staining; unaffected cells are pale gray or dark blue.

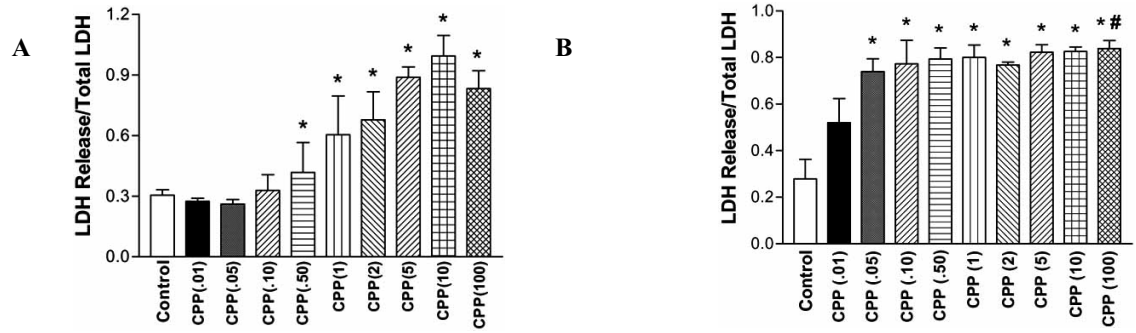

Fig. (4). Cytotoxicity (LDH Release/Total LDH) in OK cells pre-incubated in the absence of FBS and then exposed to 0.01-100 μM CPP for 2 (**A**) or 24 (**B**) hours. There are significant increases (*p< 0.05) in cytotoxicity in cells exposed to concentrations of CPP at least 0.05 μM versus the control condition in after exposure for 2 or 24 hours. Cells exposed to higher concentrations (at least 5 μM) of CPP for either 2 or 24 hours also exhibited significant differences (# p< 0.05) in toxicity compared to lower concentrations of CPP.

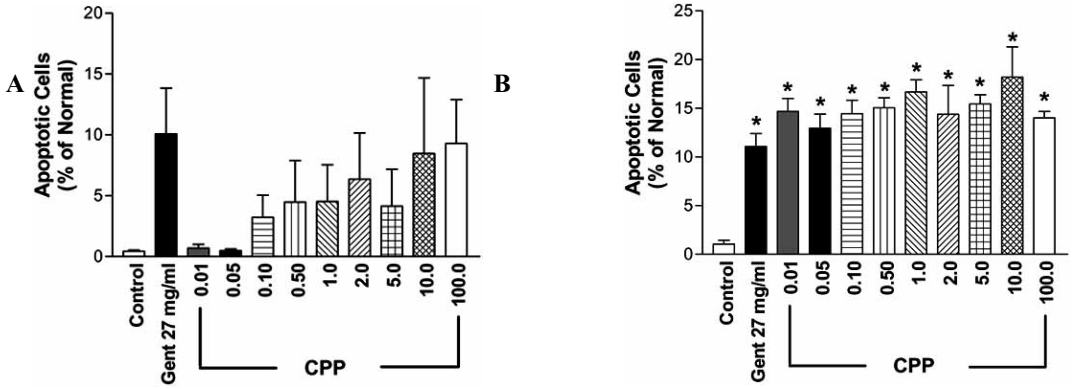

Fig. (5). Apoptosis in OK cells exposed to 0.01-100 μM CPP. CPP was applied to the cells for either 2 hours (**A**) or 24 hours (**B**), and then Hoechst stain 33258 was added to the cells. Apoptotic cells stain white and are easily visible; normal cells are identified by gray staining, and on this reproduction are not easily visible. There is a time and dose-dependent increase in apoptosis in the cells. As a positive control, the cells were exposed to high-dose gentamicin (27 mg/ml).

of receptors in a specific cell type also may determine the affect of antagonists on cell function." Finally, the concentrations of MK-801 and CPP we used were below or similar to those used to block the CNS NMDAR [25,26,27, 30,31,33]. In addition, the time period of exposure of OK cells to the antagonists was similar to that used in prior studies [6,8,35,24-26,27-32,35,36].

Our prior *in vivo* studies showed MK-801 protected the whole kidney from gentamicin nephrotoxicity [11]. Thus, there seems to be a discrepancy between the actions of

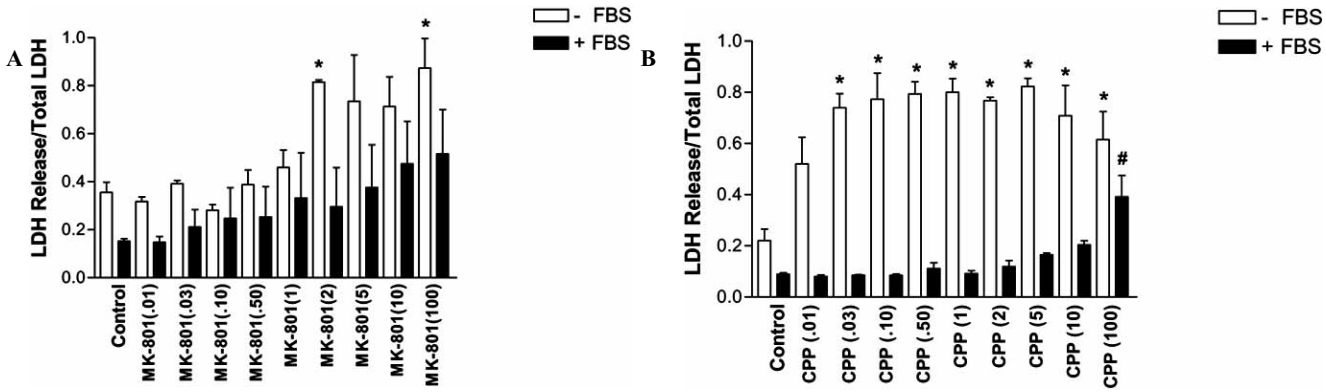

Fig. (6). Cytotoxicity (LDH Release/Total LDH) in OK cells pre-incubated in the absence (open bars) or presence (closed bars) of FBS and then exposed to 0.01-100 μM MK-801 (**A**) or 0.01-100 μM CPP (**B**) for 24 hours. There are significant increases (*p< 0.05) in cytotoxicity in cells exposed to concentrations of both MK-801 and CPP versus the control condition in FBS-deplete cells; although toxicity was higher in FBS-replete cells exposed to high (>5 μM) concentrations of either MK-801 or CPP, only the highest concentration of CPP induced a significant increase in toxicity (#p<0.05 versus control).

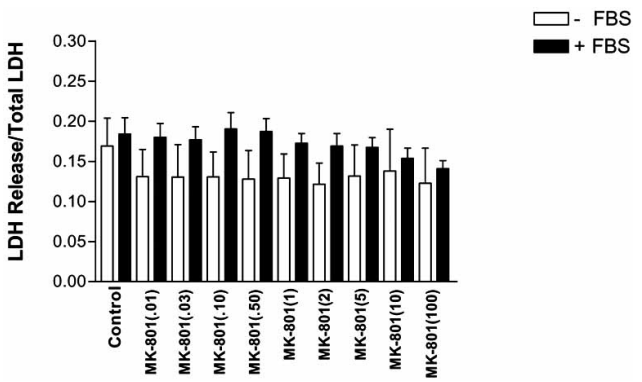

Fig. (7). Cytotoxicity (LDH Release/Total LDH) in MDCK cells pre-incubated in the absence of FBS and then exposed to 0.01-100 μM MK-801 for 24 hours. There are no differences in cytotoxicity in cells exposed to any concentration of MK-801 versus the control condition.

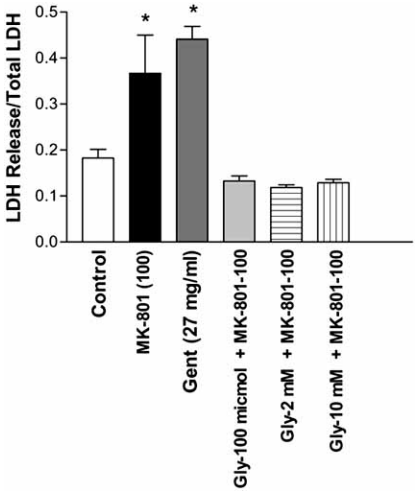

Fig. (8). Cytotoxicity (LDH Release/Total LDH) in OK cells pre-incubated in the absence of FBS and then pre-treated with low (100 μM), moderate (2 mM) or high (10 mM) dose glycine, and then exposed to a high dose (100 μM) MK-801 for 24 hours. Compared to control, 100 μM MK-801 and high-dose (27 mg/ml) gentamicin induce significantly toxicity. MK-801-induced toxicity is completely blunted in cells pre-treated with all doses of glycine.

NMDAR antagonists *in vitro* and *in vivo*. In the *in vivo* model we injected MK-801 intraperitonealy whereas in the current studies the antagonists were added directly to the cells. We hypothesize that the lack of toxicity induced by NMDAR antagonists in the whole animal model was due, at least in part, to the lower dose delivered to the proximal tubule. Additionally, the period of exposure was also different in these two models. Furthermore, the underlying condition of the cell or the cell type may influence the effect of NMDAR antagonists. For example, Priestley *et al.* previously showed that MK-801 concentrations similar to those we used (0.03-0.10 μM) protected rat cortical cells against hypoxia-induced toxicity, whereas the same concentrations in our current studies induced significant cell damage in OK cells. The differences between their study and ours may be due to several factors. First, we did not induce hypoxia in our cells and therefore studied OK cells under normal basal conditions. Second, the expression and composition of the NMDA receptor subunits may be different between the two cell types.

Based on studies showing that glycine attenuates renal toxicity caused by various nephrotoxicants [21-23] we hypothesized that NMDAR antagonist-induced renal cell death may be attenuated by pre-treatment with glycine. Our studies show that cells exposed to high-dose MK-801 exhibited significant cell toxicity; this was completely blunted by pre-treatment of the cells with glycine.

There are several limitations to the interpretation of our results. First, despite molecular evidence that the NMDAR is expressed in the kidney [9,10], it is not yet clear if the renal NMDAR is a functional receptor. Our current studies were not designed to answer that important question, but the current data and prior studies performed in our laboratory do show that the expression of the receptor correlates with cell damage [11]. We also did not perform *in vivo* experiments, which may have enhanced the significance of the current data, but it was our intent to specifically study the effect of NMDAR agonists and antagonists in the proximal tubule, and we believed this was best achieved using the cell culture model. Another concern with our results is the absolute concentration of glutamate required to induce toxicity in OK cells compared to the CNS. We only observed toxicity with very high (greater than 1 mM) doses of glutamate. This suggests that compared to the CNS NMDAR renal cells appear to be less sensitive to glutamate, or alternatively, the receptor is less abundantly expressed. This raises some legitimate concerns regarding the relative importance of the receptor in the kidney.

In summary, we have shown that the NMDAR agonist glutamate incurs damage whereas glycine alone has no effect on renal cell survival. FBS had a protective effect on MK-801 and CPP induced cell toxicity, possibly due to the presence of growth factors or phosphorylation of proteins that protect against toxicity. Finally, pre-treatment of cells with glycine completely protected against cell death. Based on current knowledge about the function of NMDA receptors in the CNS, we hypothesize that the renal NMDAR receptor may perform basal functions necessary for renal cell survival.

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