European Journal of Pharmacology 610 (2009) 12–17

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Neuroprotective effects of mebudipine and dibudipine on cerebral oxygen–glucose deprivation/reperfusion injury

Bahareh Tavakoli-Far, Nahid Rahbar-Roshandel, Parvaneh Rahimi-Moghaddam, Massoud Mahmoudian *

Razi Institute For Drug Research, Department of Pharmacology, Iran University of Medical Science, Tehran, Iran

ARTICLE INFO

Article history:
Received 3 January 2009
Received in revised form 25 February 2009
Accepted 3 March 2009
Available online 12 March 2009

Keywords:
Mebudipine
Dibudipine
Nitric oxide
Oxygen–glucose deprivation
Cortical neuron culture
Excitotoxicity

ABSTRACT

In the present study, we investigated the effects of mebudipine and dibudipine, two new Ca^{2+} channel blockers, on primary murine cortical neurons exposed to oxygen–glucose deprivation/reperfusion. The experiments were performed on cells after 11–16 days of culture. To initiate oxygen–glucose deprivation / reperfusion, the culture medium was replaced by glucose-free medium, and the cells were transferred to a humidified incubation chamber in a mixture of 95% N_2 and 5% CO_2 at 37 °C for 30 min. The cultures were pretreated with mebudipine and dibudipine 3 h prior to oxygen–glucose deprivation/reperfusion, in order to explore their effects on neurons under oxygen–glucose deprivation conditions. Cell viability and nitric oxide (NO) production were assessed by MTT assay and the modified Griess method, respectively. Exposure of murine cortical neuronal cells to 30 min oxygen–glucose deprivation significantly decreased cell viability and increased NO production. Pretreatment of the cultures with mebudipine and dibudipine significantly increased cell viability and decreased NO generation in a dose-dependent manner. However, the drugs had no protective effect in cells subjected to oxygen–glucose deprivation for 60 min. Pretreatment of cultures with MK-801 (10 μM), a non-competitive NMDA antagonist, decreased neuronal death after 30-min oxygen–glucose deprivation, while application of NBQX (30 μM), a selective AMPA-kainate receptor antagonist, partially attenuated the cell injury. oxygen–glucose deprivation -induced cytotoxicity and NO production were also inhibited by N-nitro-L-arginine methyl ester (L-NAME), a nitric oxide synthase inhibitor and MK-801. We conclude that mebudipine and dibudipine could protect cortical neurons against oxygen–glucose deprivation /reperfusion-induced cell injury in a dose-dependent manner, and that this could be mediated partially by decreased NO production.

1. Introduction

During hypoxia/ischemia of the central nervous system, the lack of energy and ATP in the brain may result in the depolarization of neurons (Heiss and Graf, 1994) that release large amounts of excitatory neurotransmitters, such as glutamate (Globus et al., 1988; Siesjo, 1992a,b). Energy-dependent processes such as presynaptic reuptake of excitatory amino acids are therefore impeded, further increasing the accumulation of glutamate in the extracellular space. Acute elevation of glutamate activates glutamate receptors, including the N-methyl-D aspartate (NMDA) receptor, which is a highly Ca2+-permeable, ligand-gated ion channel, and α-amino-3-hydroxy-5-methylisoxazole-4-propionate (AMPA, kainate) receptors, which are clearly implicated in neurotoxicity (Ban et al., 2008). Many studies have referred to NMDA receptor-triggered, Ca^{2+}-mediated excitotoxicity as "rapidly triggered", emphasizing the speed at which it can occur. In cortical cell cultures, 3–5 min of sustained NMDA receptor activation is sufficient to destroy most neurons. In contrast, AMPA receptor-triggered, Ca^{2+}-mediated excitotoxicity typically occurs more slowly, requiring hours of sustained receptor activation to induce lethal injury in the same cell cultures (Simeone et al., 2004). Excessive stimulation of the NMDA receptor following hypoxia/ischemia leads to excessive intracellular Ca^{2+} influx that can activate Ca^{2+}-dependent degradative enzymes such as proteases, nucleases, phospholipases, and nitric oxide synthase (NOS) (Gunasekar et al., 1995). The rise in Ca^{2+} can activate neuronal NOS (nNOS)/mitochondrial NOS (mtNOS), and generate nitric oxide (NO), which directly contributes to toxicity (Brorson et al., 1994; Ghafourifar et al., 1999; Horn et al., 2002; Jekabsone et al., 2003).

The generation of reactive oxygen species such as hydroxyl radicals, superoxide anions and NO can cause lipid peroxidation and membrane damage (Lovs, 1999), and eventually neuronal apoptosis or necrosis, depending on the intensity of the initial insult and the extent of energy recovery (Gagliardi, 2000). However, NO and free radicals inhibit Ca^{2+}-ATPases and release Ca^{2+} from the endoplasmic reticulum, suggesting that endoplasmic reticulum Ca^{2+} could play a role in oxidant toxicity (Gepdiremen et al., 2002). Therefore, glutamate-induced oxidative stress during hypoxia/ischemia plays a

* Corresponding author. Tel.: +98 21 88058696; fax: +98 21 88052978.
E-mail address: masmah99@iums.ac.ir (M. Mahmoudian).

0014-2999/$ – see front matter © 2009 Published by Elsevier B.V.
doi:10.1016/j.ejphar.2009.03.017

critical role in excitotoxicity (Warner et al., 2004) that leads to neuronal death (Yoshioka et al., 2000) and impaired brain function (Berman et al., 2000).

L-type voltage-sensitive Ca^{2+} channels mediate long-lasting Ca^{2+} currents in response to depolarization in excitable cells (Yagami et al., 2004). Several studies using models of cerebral ischemia have reported neuroprotective actions of Ca^{2+} channel blockers. For example, L-type Ca^{2+} channel blockers, such as S-emopamil, nimodipine and nicardipine, have been shown to display neuroprotective effects in some animal models of ischemia (Alps, 1992; Lin et al., 1990; Morikawa et al., 1991; Rami and Krieglstein, 1994). Various classes of Ca^{2+}-channel blockers have been introduced (Fleckenstein, 1977): 1,4-dihydropyridine (DHP) derivatives have widely demonstrated the existence of L-type Ca^{2+}channels. Mebudipine ((±)-bis-t-butyl-1,4-dihydro-2,6-dimethyl-1,4-(3-nitrophenyl)-3,5-pyrinedicarboxylate) and dibudipine ((±)-bis-t-butyl-1,4-dihydro-2,6-dimethyl-1,4-(3-nitrophenyl)-3,5-pyridine dicarboxylate) are two new Ca^{2+} channel blockers with DHP structures. Both compounds were first synthesized by Mahmoudian et al., (Mahmoudian et al., 1997). The metabolism of these Ca^{2+} channel blockers in isolated rat hepatocytes has been studied under in vitro conditions (Bohlooli et al., 2004) and they were shown to be metabolized extensively through oxidative pathways and by O-glucuronidation. Several studies have evaluated their effects on the contractions of various tissues. Both compounds relaxed isolated human internal mammary artery rings, with mebudipine having greater potency than dibudipine. They also reduced the contractile force of the rat left atrium, suggesting that mebudipine might have a selective and protective Ca^{2+} channel blocking effect (Mirkhani et al., 1999, 2005). Another study showed that mebudipine and dibudipine were potent relaxants of vascular and ileal smooth muscle, and that their potencies in these processes were comparable with those of nifedipine (Mahmoudian et al., 1997).

We previously-reported the protective effect of nimodipine on oxygen–glucose deprivation-induced and glutamate-induced neurotoxicity in PC12 cells (Rahbar-Roshandel et al., 2007, 2008). This study aimed to test if mebudipine and dibudipine, two new Ca^{2+} channel blockers, could protect cortical neuronal cells against oxygen–glucose deprivation /reperfusion-induced neurotoxicity and NO production, and to compare their neuroprotective abilities with that of nimodipine.

2. Materials and methods

2.1. Chemicals and reagents

Mebudipine and dibudipine were synthesized in our laboratories (Mahmoudian et al., 1997); nimodipine, RPMI-1640, Dulbecco's modified Eagle's medium (DMEM) without glutamine, fetal bovine serum (FBS), horse serum, penicillin–streptomycin, cytosine arabinoside, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), poly-L-lysine, dizocilpine (MK-801), NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione), N^G nitro-L-arginine methyl ester (L-NAME), and modified Griess reagent were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals were obtained from Merck Co. (Darmstadt, Germany).

2.2. Murine neuronal cell cultures

Primary neuronal cultures of cerebral cortex were obtained from mouse embryos (E_{15-18}), as described previously (Dawson et al., 1991, 1993). Briefly, timed-pregnant female mice were anesthetized with chloroform and killed by cervical dislocation. Fetuses were removed and decapitated with small scissors. The skin and skull were removed, the cerebral cortex was isolated and the cortex was cut into small pieces. Tissue was then dissociated by repeated pipetting. The resulting homogenate was centrifuged at 300 ×*g* for 5 min at 20 °C and plated on poly-L-lysine-coated 96-well plate culture dishes at a density of 5×10^4 cells/ml in RPMI-1640 medium supplemented with 10% FBS, 5% horse serum, and 100 IU/ml penicillin and 100 µg/ml streptomycin. Poly-L-lysine (150,000–300,000 MW) was dissolved in deionized water and the plate bases were filled with this solution (20 µg/ml) 24 h before the experiment. After 5 min standing at room temperature, the solution was vacuumed and the plates were left to dry in a laminar flow bench overnight. The culture dishes were kept at 37 °C in humidified 95% air and 5% CO_2. After 24–48 h, 10 µM cytosine arabinoside (cytosine 1-β-D-arabinofuranoside) was added to the culture medium to prevent the proliferation of non-neuronal cells. The culture medium was changed twice weekly, and the neurons were used for experiments after 11–16 days incubation. All experiments were approved by the Institute of Animal Care Committee at Iran University of Medical Sciences.

2.3. Oxygen–glucose deprivation and drug exposure

Procedures for oxygen–glucose deprivation were performed as described previously (Frantseva et al., 1999). Briefly, the culture medium was replaced with glucose/glutamine-free DMEM, and was exposed to hypoxia for 30 or 60 min in a small anaerobic chamber previously filled with 95% (v/v) N_2 and 5% (v/v) CO_2 at 37 °C. To terminate the oxygen–glucose deprivation, the chamber was opened and the medium was replaced with RPMI-1640, and the cultures were then placed in an incubator with 5% CO_2 for 24 h. To examine the drug effects, cell cultures were treated 3 h before oxygen–glucose deprivation with mebudipine, dibudipine (dissolved in dimethylsulfoxide (DMSO)) or nimodipine (dissolved in methanol), as a positive control, at concentrations of 10^{-10}, 10^{-9}, 10^{-8}M. These concentrations were chosen for the drugs based on the results of preliminary experiments (data not shown). The pre-incubation time of 3 h was selected based on a previously-reported data (Rahbar-Roshandel et al., 2008). To investigate the effects of various inhibitors on oxygen–glucose deprivation-induced cell death, MK-801, a non-competitive antagonist of the NMDA receptor, NBQX, an AMPA receptor antagonist, and L-NAME, a NOS inhibitor, were added to the medium 3 h before oxygen–glucose deprivation. All measurements were repeated eight times and each experiment was repeated at least three times. Every data point is therefore the mean of at least 24 measurements.

2.4. Analysis of neuronal cell viability

Neuronal cell viability was monitored using the colorimetric MTT assay, as previously described (Mosmann, 1983). Briefly, cells were incubated with 0.5 mg/mL MTT in RPMI, at 37 °C under 5% CO_2, for 3 h. The blue formazan reduction product, produced by the action of succinate dehydrogenase in living cells on the dye, was dissolved in 100 µL DMSO, and the optical density was read at 570 nm using a Dynex MMX microplate reader (Dynex, Richfield, MN, USA).

Data were expressed as the percentage of viable cells in oxygen–glucose deprivation-exposed plates compared with control normoxic plates determined by MTT reduction.

2.5. Measurement of nitrite

The level of nitrite as a measure of NO production in the culture medium was measured using modified Griess reagent. In brief, after the experiment, the medium in each well was removed and centrifuged at 10,000 ×*g* for 10 min at 20 °C. Then, 100 µL of the supernatant were mixed with an equal volume of Griess reagent at room temperature for 10 min, and the absorbance was measured at 540 nm using a microplate reader. The nitrite concentration was determined from a sodium nitrite standard curve. Plates not exposed to oxygen–glucose deprivation were used as an external control and those exposed to oxygen–glucose deprivation conditions without the addition of any drug were used as an internal control.

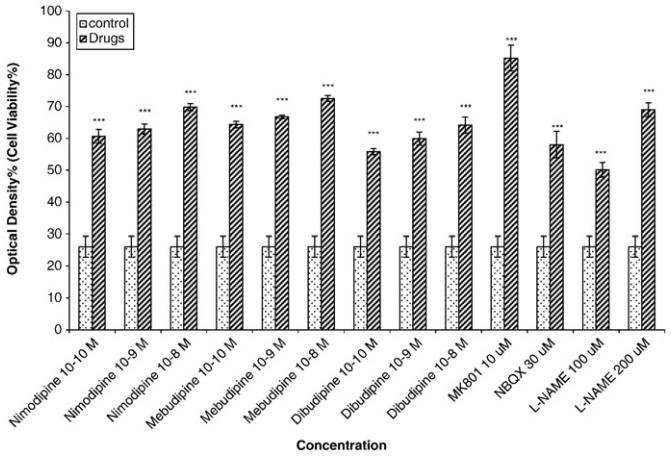

Fig. 1. The effects of mebudipine and dibudipine in comparison with nimodipine, MK801, NBQX, and L-NAME on a 30-min oxygen–glucose deprivation/24 h reperfusion-induced cell injury in cultured murine cortical neurons. The dashed bar shows the drugs group and the dotted bar shows the control group. Optical density at 570 nm corresponds to the blue formazan reduction product which is produced by the action of mitochondrial succinate dehydrogenase in living cells and correlates to cell viability. Data are expressed as the percentage of viable cells in oxygen–glucose deprivation-exposed plates compared with control normoxic plates (100%). ***$P<0.001$.

2.6. Statistical analysis

Data are expressed as means ± S.E.M. The significance of differences between means was determined using Student's *t*-test. The *P* values <0.05 were considered significant.

3. Results

3.1. The effects of mebudipine and dibudipine, compared with nimodipine, on oxygen–glucose deprivation-induced cell injury and NO production, in cultured cortical neurons

Oxygen–glucose deprivation conditions for 30 or 60 min, followed by 24 h reperfusion, caused significant cell death, as shown in Fig. 1. We examined the effects of two new L-type voltage-gated Ca^{2+} channel blockers, mebudipine and dibudipine, on 30- and 60-min oxygen–glucose deprivation -induced neurotoxicity in cortical neurons. Nimodipine was assessed as a positive control. Pretreatment of cultures with mebudipine (10^{-10}, 10^{-9}, 10^{-8}M), dibudipine (10^{-10}, 10^{-9}, 10^{-8}M), or nimodipine (10^{-10}, 10^{-9}, 10^{-8}M), 3 h before exposure to 30 min oxygen–glucose deprivation /reperfusion significantly increased the percentage of viable cells, in a concentration-

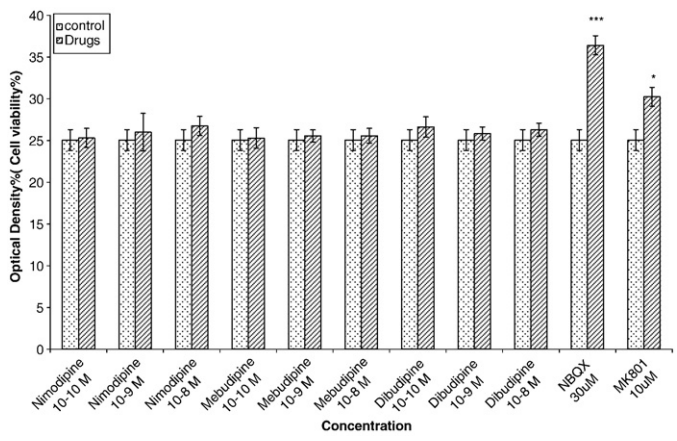

Fig. 2. The effects of mebudipine and dibudipine in comparison with nimodipine, MK801, and NBQX, on 60 min oxygen–glucose deprivation/24 h reperfusion-induced cell injury in cultured murine cortical neurons. The dashed bar shows the drugs group and the dotted bar shows the control group. Optical density at 570 nm corresponds to the blue formazan reduction product which is produced by the action of mitochondrial succinate dehydrogenase in living cells and correlates to cell viability. Data are expressed as the percentage of viable cells in oxygen–glucose deprivation-exposed plates compared with control normoxic plates (100%). ***$P<0.001$.

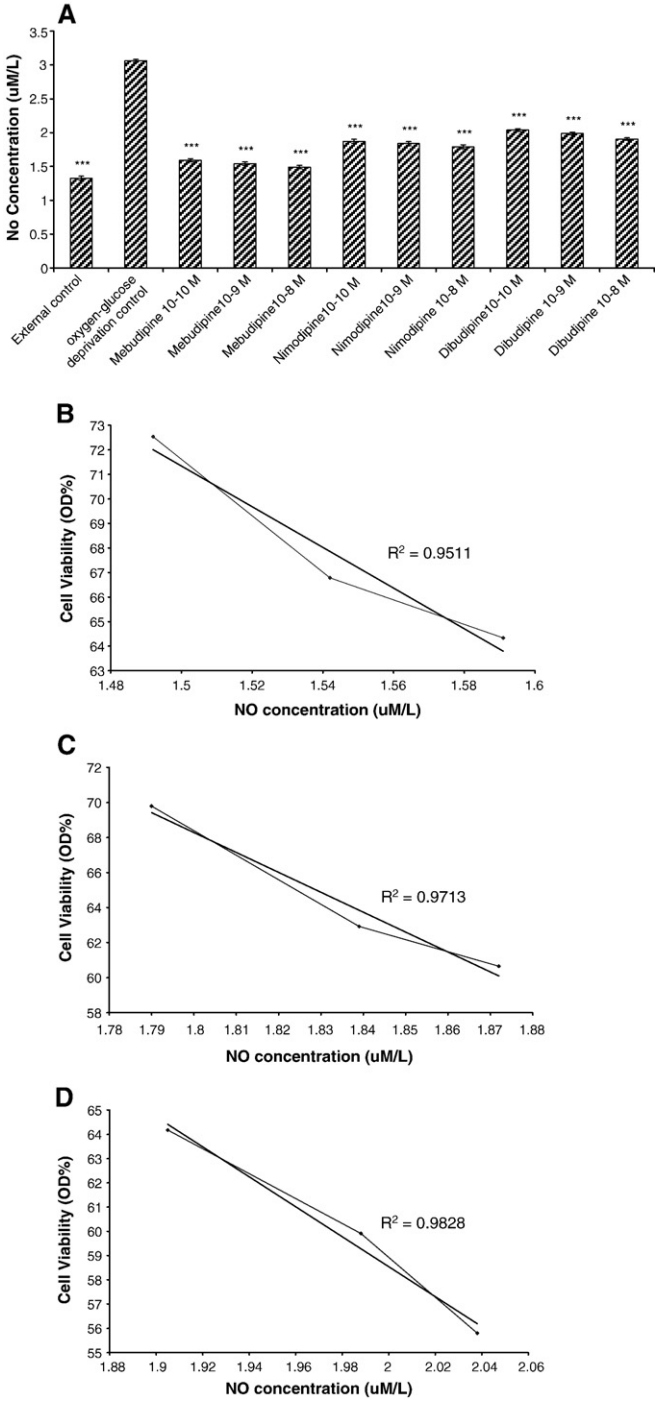

Fig. 3. (A) The effects of mebudipine and dibudipine in comparison with nimodipine on NO production during a 30-min oxygen–glucose deprivation/24 h reperfusion-induced cell injury in cultured murine cortical neurons. ***$P<0.001$ (B) The correlation between NO production and mebudipine-induced cell survival after a 30-min oxygen–glucose deprivation/24 h reperfusion. (C) The correlation between NO production and nimodipine-induced cell survival after a 30-min oxygen–glucose deprivation/24 h reperfusion. (D) The correlation between NO production and dibudipine-induced cell survival after a 30-min oxygen–glucose deprivation/24 h reperfusion. The values are presented as mean ± S.E.M.

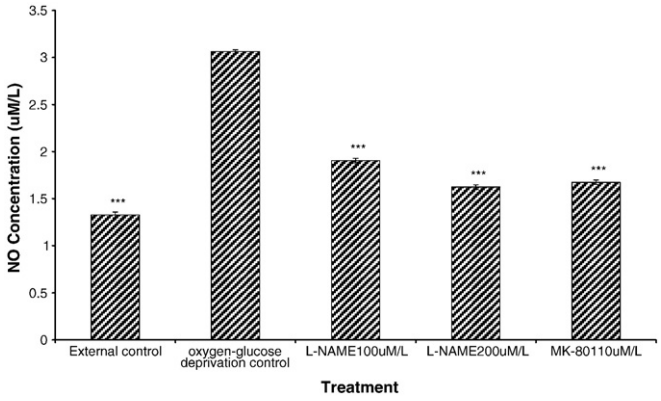

Fig. 4. The effects of L-NAME on NO production during a 30-min oxygen–glucose deprivation/24 h reperfusion-induced cell injury in cultured murine cortical neurons.

dependent manner (Fig. 1). The most effective dose was 10^{-8}M. These drugs had no protective effects in 60 min oxygen–glucose deprivation (Fig. 2).

Exposure of cells to 30 min of oxygen–glucose deprivation/reperfusion significantly increased nitrite production ($P<0.001$), as shown in Fig. 3A. Pretreatment of cultures with mebudipine, dibudipine or nimodipine (10^{-10}, 10^{-9}, 10^{-8}M) significantly decreased NO production, as compared with the external control.

There was a highly negative correlation between cell viability and NO production during oxygen–glucose deprivation after pretreatment with mebudipine, dibudipine and nimodipine (Fig. 3B–D).

3.2. The effects of MK-801 and NBQX on oxygen–glucose deprivation-induced cell injury in cultured cortical neurons

In order to determine which glutamate receptors were responsible for neuronal cell loss induced by 30 min of oxygen–glucose deprivation, cultures were pretreated with glutamate receptor antagonists, including MK-801 (10 μM) and NBQX (30 μM). MK-801, a non-competitive NMDA receptor antagonist, significantly and markedly (85%) increased cell viability, but NBQX, a selective AMPA-kainate receptor antagonist, only partially (58%) prevented the cell death induced by oxygen–glucose deprivation (Fig. 1). In a 60-min oxygen–glucose deprivation, the same concentrations of MK-801 and NBQX increased cell viability by 30% and 36% respectively.

3.3. The effect of L-NAME and MK-801 on oxygen–glucose deprivation-induced cell injury and NO production

The effects of the NOS inhibitor, L-NAME (100 or 200 μM) and MK-801 (10 μM) on cell viability and NO production were also examined. Oxygen–glucose deprivation-induced neuronal death and NO production were attenuated by L-NAME and MK-801 (Fig. 4).

4. Discussion

Ca^{2+} channel blockers have neuroprotective potential against high glutamate- and oxygen–glucose deprivation/reperfusion-induced cell injury in neuronal cell cultures (Zhang et al., 1993). For example, nimodipine, a DHP voltage-gated Ca^{2+} channel blocker, significantly reduced Ca^{2+}influx and demonstrated a dose-dependent neuroprotective effect in kainate-, glutamate-, and NMDA-induced neurotoxicity in rat cerebellum (Gepdiremen et al., 1997, 2001; Duzenli et al., 2005). In the present study, we investigated if the two new Ca^{2+} channel blockers, mebudipine and dibudipine (Mahmoudian et al., 1997), were able to protect cortical neuronal cells against 30- and 60-min oxygen–glucose deprivation/reperfusion, compared with nimodipine. Our results indicate that mebudipine and dibudipine, like nimodipine, significantly attenuated neuronal cell death after 30 min of oxygen–glucose deprivation /reperfusion in primary cultures of mouse cortical neurons, in a concentration-dependent manner. When the oxygen–glucose deprivation period was increased to 60 min, however, the Ca^{2+} channel blockers had no protective effects on the neuronal cells. The protective effect of these L-type Ca^{2+}-channel blockers is confirmed by their involvement in reduction of NO production, as NO is an important mediator in neuronal cell death (Dawson et al., 1996).

During hypoxia/ischemia, activation of NMDA glutamate receptors elevates the influx of Ca^{2+}, and activation of non-NMDA receptors promotes the influx of Na^{+}, both of which can lead to membrane depolarization. Depolarization can, in turn, activate plasma membrane voltage-sensitive Ca^{2+} channels, leading to additional Ca^{2+} influx (Heiss and Graf, 1994). It is likely that glutamate acts on AMPA receptors to produce membrane depolarization and sufficient membrane depolarization removes the Mg^{2+} block of NMDA channels, allowing the influx of extracellular Ca^{2+} into the cell (Mayer et al., 1984; Lin et al., 2008). Released glutamate acts on glutamate receptors and thereby potentiates the neurotoxicity. Blocking of Ca^{2+}-permeable NMDA receptors markedly reduces the rise in intracellular Ca^{2+} (Silver and Erecinska, 1990) and has been shown to be neuroprotective in animal models of focal brain ischemia, hypoglycemia, and trauma (Choi, 1988; Albers et al., 1989). However, application of AMPA-kainate receptor antagonists did not afford complete neuroprotection in oxygen–glucose deprivation conditions, despite reducing the glutamate efflux to a large extent (Kimura et al., 1998). To investigate the role of glutamate receptors, we examined if MK-801, a non-competitive NMDA receptor antagonist, and NBQX, a potent competitive inhibitor of AMPA/kainate receptors, could attenuate oxygen–glucose deprivation/reperfusion-induced cell death in primary cortical cultured neurons. Our results indicated that MK-801 had greater inhibitory effects on cell death induced by 30 min of oxygen–glucose deprivation/reperfusion, while NBQX only partially inhibited cell death induced by oxygen–glucose deprivation/reperfusion. In 60-min oxygen–glucose deprivation, application of the same concentrations of MK-801 and NBQX resulted in a more prominent protective effect of NBQX than that of MK-801. This is consistent with previous reports indicating that on cortical neurons, AMPA-kainate receptors modulate a more slowly-triggered excitatoric injury than NMDA receptors do. (Frandsen et al., 1989; Koh et al., 1990). In fact, NMDA over-stimulation may mask AMPA-induced injury. These results suggest that non-NMDA receptors are partially involved in cell death during oxygen–glucose deprivation /reperfusion, but that NMDA receptor activation and subsequent Ca^{2+} influx is the primary cause of cell injury and play crucial roles in cell death. This is consistent with previous studies which suggested that increased levels of glutamate and the subsequent activation of ionotropic NMDA receptors were primarily responsible for neuronal damage occurring as a consequence of ischemic or hypoxic episodes (Simon et al., 1984; Rothman and Olney, 1986; Sattler et al., 2000; Ban et al., 2008).

Our current study has demonstrated that L-type Ca^{2+} channel blockers (mebudipine, dibudipine, and nimodipine), like MK-801 could protect cortical neurons against a 30-min oxygen–glucose deprivation/reperfusion-induced cell death. This suggests that inhibition of both NMDA receptor-gated and L-type voltage-dependent Ca^{2+} channels results in neuroprotection which is probably due to decreased Ca^{2+} influx into the cells. In turn, the drugs had no neuroprotective effect during 60 min of oxygen–glucose deprivation/reperfusion. These results are confirmed by the fact that cell death induced by short-term oxygen–glucose deprivation/reperfusion is Ca^{2+}-dependent, but during longer periods of oxygen–glucose deprivation/reperfusion, additional factors could be involved in enhancing cell death (MacDonald and Jackson, 2007). There are lots of controversies in the role of the various ionic pathways involved in ischemic neurotoxicity. Martinez-Sánchez et al. (2004) in a systemic study on

hippocampal slices showed that oxygen–glucose deprivation-induced increase in intracellular calcium might be caused by simultaneous activation of NMDA receptors, Na^+/Ca^{2+} exchangers and Na^+ channels. The same group did not observe any involvement of voltage-sensitive Ca^{2+} channels, intracellular Ca^{2+} stores and AMPA-kainate receptors in increased intracellaular calcium. In another study, no neuroprotective effect was detected for p-type Ca^{2+} channels blockers (Lingenhohl et al., 1997). Also, Pringle et al. (1996) showed that L-type Ca^{2+} channel blockers did not protect hippocampal-slice cultures exposed to 1 h of oxygen–glucose deprivation. In the other hand, several studies have shown the protective effect of L-type Ca^{2+} channel blockers in oxygen–glucose deprivation-induced neuron injury (Pisani et al., 1998; Duzenli et al., 2005; Zhang et al., 2008).

For the measurement of cellular NO production, direct measurement of released NO by electrodes or measurement of itsmetabolites-nitrite by Griess method has been used (Kimura et al., 2001). In this study, the modified Griess method was used to investigate the roles of mebudipine and dibudipine on NO production induced by short-term oxygen–glucose deprivation /reperfusion. The results showed that NO production was significantly increased during 30-min oxygen–glucose deprivation /reperfusion, supporting a role for NO in the cascade of events leading to a 30-min oxygen–glucose deprivation/reperfusion-induced cell death. Interestingly, when the cultures were pretreated with mebudipine, dibudipine or nimodipine, the NO production was significantly decreased and returned to the level of the external control. We observed that both L-NAME, a nNOS inhibitor, and MK-801, a non-competitive NMDA antagonist increased cell viability and significantly decreased NO production. The same results have been reported in a previously-published study which shows that application of MK-801 significantly blocked the oxygen–glucose deprivation-induced increases of intracellular calcium and NO (Zhang et al., 2003). Previous studies have also indicated that NOS inhibitors could markedly reduce the generation of NO after NMDA receptor activation, and decreased excitotoxic injury in cultured neurons (Moncada et al., 1992; Kowara et al., 2006). Several studies have found evidence indicating an involvement of NOS in glutamate-induced excitotoxicity (Dawson et al., 1991; Shibuta et al., 2003; Whiteman et al., 2004). It was proposed that NMDA receptor-mediated excitotoxicity involved Ca^{2+}/calmodulin-dependent NOS activation, and generation of NO and superoxide (Chakravarthy et al., 1999; Dawson et al., 1996; Guix et al., 2005; Weaver et al., 2005). Another report indicated that a low concentration of NO played a protective role in glutamate neurotoxicity by closing the NMDA receptor-gated ion channels, while high concentrations of NO interacted with oxygen radicals, becoming toxic and mediating glutamate-induced neurotoxicity in cultured retinal neurons. NO may therefore possess both neurodestructive and neuroprotective properties (Gepdiremen et al., 2002). The relevance of NO to neurotoxicity is also confirmed by the results of a study reporting that the diffusion-limited reaction between superoxide and NO gave rise to peroxynitrite (Beckman et al., 1990). The highly reactive peroxynitrite provides a mechanistic basis for oxidative stress derived from increased NO production caused by ischemia/reperfusion (Eliasson et al., 1999). NMDA/AMPA/kainate receptor activation evokes a rise in cytosolic Ca^{2+}, activating nNOS along with a high capacity, ruthenium red-sensitive Ca^{2+} uniporter that transports Ca^{2+} into the mitochondria, which in turn activates mtNOS (Cadenas et al., 2001; Haynes et al., 2003). Activation of nNOS/mtNOS can evoke a rise in NO/peroxynitrite leading to cell death (Ghafourifar et al., 1999; Sanchez-Gomez et al., 2003). We have shown that mebudipine and dibudipine, like nimodipine, could return the NO production to control levels. The increase in NO production induced by a 30-min oxygen–glucose deprivation might be partially due to activation of the nNOS isoform in these cells. In fact, we can suggest that during oxygen–glucose deprivation, calcium enters into neurons through NMDA receptor and/or L-type voltage-gated calcium channels and activates NOS which leads to NO production.

In conclusion, mebudipine and dibudipine, two new L-type Ca^{2+} channel blockers, were able to protect cortical neurons against 30 min of oxygen–glucose deprivation via a decrease in NO production. This was confirmed by the observation that there was a highly negative correlation between decreased NO production and cell death during 30 min of oxygen–glucose deprivation/reperfusion. These results suggest an order of efficacy of mebudipine > nimodipine > dibudipine. These observations support the hypothesis of a critical role for the Ca^{2+} current during ischemic insult. Therefore, blockade of calcium influx through NMDA receptor-coupled channels and voltage-dependent calcium channels might be an effective approach for neuronal protection during oxygen–glucose deprivation.

References

Albers, G.W., Goldberg, M.P., Choi, D.W., 1989. *N*-Methyl-D-aspartate antagonists: ready for clinical trial in brain ischemia? Ann. Neurol. 25, 398–403.

Alps, B.J., 1992. Drugs acting on calcium channels: potential treatment for ischemic stroke. Br. J. Clin. Pharmacol. 34, 199–206.

Ban, J., Cho, S., Choi, S., Ju, H., Kim, J., Bae, K., Song, K., Seong, Y., 2008. 1Neuroprotective effect of *Smilacis chinae* rhizome on NMDA-induced neurotoxicity in vitro and focal cerebral ischemia in vivo. J. Pharmacol. Sci. 106, 68–77.

Beckman, J.S., Beckman, T.W., Chen, J., Marshall, P.A., Freeman, B.A., 1990. Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1624.

Berman, R.F., Verweij, B.H., Muizelaar, J.P., 2000. Neurobehavioral protection by the neuronal calcium channel blocker ziconotide in a model of traumatic diffuse brain injury in rats. J. Neurosurg. 93, 821–828.

Bohlooli, S., Mahmoudian, M., Skellern, G.G., Grant, M.H., Tettey, J.N., 2004. Metabolism of the dihydropyridine calcium channel blockers mebudipine and dibudipine by isolated rat hepatocytes. J. Pharm. Pharmacol. 56, 1469–1475.

Brorson, J.R., Manzolillo, P.A., Miller, R.J., 1994. Ca^{2+} entry via AMPA/KA receptors and excitotoxicity in cultured cerebellar Purkinje cells. J. Neurosci. 14, 187–197.

Cadenas, E., Poderoso, J.J., Antunes, F., Boveris, A., 2001. Analysis of the pathways of nitric oxide utilization in mitochondria. Free Radic. Res. 33, 747–756.

Chakravarthy, B., Morley, P., Whitfield, J., 1999. Ca^{2+}-calmodulin and protein kinase C: a hypothetical synthesis of their conflicting convergences on shared substrate domains. Trends. Neurosci. 22, 12–16.

Choi, D.W., 1988. Calcium-mediated neurotoxicity: relationship to specific channel types and role in ischemic damage. Trends. Neurosci. 11, 465–469.

Dawson, V.L., Dawson, T.M., London, E.D., Bredt, D.S., Snyder, S.H., 1991. Nitric oxide mediates glutamate neurotoxicity in primary cultures. Proc. Nat1. Acad. Sci. U. S. A. 88, 6368–6371.

Dawson, V.L., Dawson, T.M., Bartley, D.A., Uhi, G.R., Snyder, S.H., 1993. Mechanisms of nitric oxide-mediated neurotoxicity in primary brain cultures. J. Neurosci. 13, 2651–2661.

Dawson, V.L., Kizushi, V.M., Huang, P.L., Snyder, S.H., Dawson, T.M., 1996. Resistance to neurotoxicity in cortical cultures from neuronal nitric oxide synthase-deficient mice. J. Neurosci. 16, 2479–2487.

Duzenli, S., Bakuridze, K., Gepdiremen, A., 2005. The effects of ruthenium red, dantrolen and nimodipine alone or in combination, in NMDA-induced neurotoxicity of cerebella granular cell culture of rats. Toxicol. In Vitro 19, 589–594.

Eliasson, M.J., Huang, Z., Ferrante, R.J., Sasamata, M., Molliver, M.E., Synder, S.H., Moskowitz, M.A., 1999. Neuronal nitric oxide synthase activation and peroxynitrite formation in ischemic stroke linked to neural damage. J. Neurosci. 19, 5910–5918.

Fleckenstein, A., 1977. Specific pharmacology of calcium in myocardium, cardiac pacemakers, and vascular smooth muscle. Ann. Rev. Pharmacol. Toxic. 17, 149–166.

Frandsen, A., Drejer, J., Schousboe, A., 1989. Direct evidence that excitatotoxicity in cultured neurons is mediated via N-methyl-D-aspartate (NMDA) as well as non-NMDA receptors. J. Neurochem. 53, 297–299.

Frantseva, M., Carlen, P., El-Beheiry, H., 1999. A submersion method to induce hypoxic damage in organotypic hippocampal cultures. J. Neurosci. Methods 89, 25–31.

Gagliardi, R.J., 2000. Neuroprotection, excitotoxicity and NMDA antagonists. Arq. Neuropsiquiatr. 58, 583–588.

Gepdiremen, A., Sonmez, S., Batat, I., Esrefoglu, M., Duzenli, S., Suleyman, H., 1997. Nimodipine improves kainic acid induced neurotoxicity in cerebellar granular cell culture: a double-blind dose-response study. Fundam. Clin. Pharmacol. 11, 117–120.

Gepdiremen, A., Duzenli, S., Hacimuftuoglu, A., Suleyman, H., Oztas, S., 2001. The study of dantrolene alone or in combination with nimodipine in glutamate neurotoxicity in cerebellar granular cell cultures of rat pups. Pharmacol. Res. 43, 241–244.

Gepdiremen, A., Hacimuftuoglu, A., Buyukokuroglu, M.E., Suleyman, H., 2002. Nitric oxide donor sodium nitroprusside induces neurotoxicity in cerebellar granular cell culture in rats by an independent mechanism from L-Type or dantrolene-sensitive calcium channels. Biol. Pharm. Bull. 25, 1295–1297.

Ghafourifar, P., Schenk, U., Klein, S.D., Richter, C., 1999. Mitochondrial nitric oxide synthase stimulation causes cytochrome c release from isolated mitochondria: evidence for intramitochondrial peroxynitrite formation. J. Biol. Chem. 274, 31185–31188.

Globus, M.Y.T., Busto, R., Dietrich, W.D., Martinez, E., Valdes, I., Ginsberg, M.D., 1988. Effect of ischaemia on the in vivo release of striatal DA, Glu and gamma-aminobutyric acid studied by intracerebral microdialysis. J. Neurochem. 51, 1455–1464.

Guix, F.X., Uribesalgo, I., Coma, M., Munoz, F.J., 2005. The physiology and pathophysiology of nitric oxide in the brain. Prog. Neurobiol. 76, 26–152.
Gunasekar, P.G., Kanthasamy, A.G., Borowitz, J.L., Isom, G.E., 1995. NMDA receptor activation produces concurrent generation of nitric oxide and reactive oxygen species: implication for cell death. J. Neurochem. 65, 2016–2021.
Haynes, V., Elfering, S.L., Squires, R.J., Traaseth, N., Solien, J., Ettl, A., Giulivi, C., 2003. Mitochondrial nitric-oxide synthase: role in pathophysiology. IUBMB Life 55, 599–603.
Heiss, W.D., Graf, R., 1994. The ischemic penumbra. Curr. Opin. Neurol. 7, 11–19.
Horn, T.F., Wolf, G., Duffy, S., Weiss, S., Keilhoff, G., MacVicar, B.A., 2002. Nitric oxide promotes intracellular calcium release from mitochondria in striatal neurons. FASEB J. 16, 1611–1622.
Jekabsone, A., Ivanoviene, L., Brown, G.C., Borutaite, V.J., 2003. Nitric oxide and calcium together inactivate mitochondrial complex I and induce cytochrome c release. Mol. Cell. Cardiol. 35, 803–809.
Koh, J., Goldberg, M.D., Hartley, D.M., Choi, D.W., 1990. Non-NMDA receptor-mediated neurotoxicity in cortical culture. J. Neurosci. 10, 693–705.
Kimura, C., Oike, M., Koyama, T., Ito, Y., 2001. Impairment of endothelial nitric oxide production by acute glucose overload. Am. J. Physiol. Endocrinol. Metab. 280, 171–178.
Kimura, M., Sawada, K., Miyagawa, T., Kuwada, M., Katayama, K., Nishizawa, Y., 1998. Role of glutamate receptors and voltage dependent calcium and sodium channels in the extracellular glutamate/aspartate accumulation and subsequent neuronal injury induced by oxygen/glucose deprivation in cultured hippocampal neurons. J. Pharmacol. Exp. Therapeut. 285, 178–185.
Kowara, R., Moraleja, K.L., Chakravarthy, B., 2006. Involvement of nitric oxide synthase and ROS-mediated activation of L-type voltage-gated Ca2+ channels in NMDA-induced DPYSL3 degradation. Brain Res. 1119, 40–49.
Lin, B., Dietrich, W.D., Busto, R., Ginsberg, M.D., 1990. *S*-emopamil protects against global brain injury in rats. Stroke 21, 1734–1739.
Lin, C.H., Chen, P.S., Gean, P.W., 2008. Glutamate preconditioning prevents neuronal death induced by combined oxygen–glucose deprivation in cultured cortical neurons. Eur. J. Pharmacol. 589, 85–93.
Lingenhohl, K., Smali, D.L., Monette, R., Buchan, A.M., Morley, P., Allegrini, P.R., Frostl, W., Sauer, D., Schmutz, M., Knopfel, T., 1997. Eploration of P-type Ca^{2+} channels as drug targets for the treatment of epilepsy or ischemic stroke. Neuropharmacology 36, 107–113.
Lovs, S., 1999. Oxidative stress in brain ischemia. Brain Pathol. 9, 119–131.
MacDonald, J.F., Jackson, M.F., 2007. Transient receptor potential channels of the melastatin family and ischemic responses of central neurons. Stroke 38, 665–669.
Mahmoudian, M., Mirkhani, H., Nehardani, Z., Ghaiee, S., 1997. Synthesis and biological activity of two new calcium-channel blockers, mebudipine, and dibudipine. J. Pharm. Pharmacol. 49, 1229–1233.
Martinez-Sánchez, M., Striggow, F., Schröder, U.H., Kahlert, S., Reymann, K.G., Reiser, G., 2004. Na^{+} and Ca^{2+} homeostasis pathways, cell death and protection after oxygen–glucose-deprivation in organotypic hippocampal slice cultures. Neuroscience 128, 729–740.
Mayer, M.L., Westbrook, G.L., Guthrie, P.B., 1984. Voltage-dependent block by Mg^{++} of NMDA responses in spinal cord neurons. Nature 309, 261–263.
Mirkhani, H., Omrani, G.R., Ghiaee, S., Mahmoudiam, M., 1999. Effect of mebudipine and dibudipine, two new calcium-channel blockers, on rat left atrium, rat blood pressure and human internal mammary artery. J. Pharm. Pharmacol. 51, 617–622.
Mirkhani, H., Dirin, M., Youssef-Zadeh, I., 2005. Mechanism of vasoselective action of mebudipine, a new calcium channel blocker. Vascular Pharmacol. 42, 23–29.
Moncada, C., Lekieffre, D., Arvin, B., Meldrum, B., 1992. Effect of NO synthase inhibition on NMDA- and ischaemia-induced hippocampal lesions. Neuroreport 3, 530–532.
Morikawa, E., Ginsberg, M.D., Dietrich, W.D., Duncan, R.C., Busto, R., 1991. Postischaemic *S*-emopamil therapy ameliorates focal ischaemic brain injury in rats. Stroke 22, 355–360.
Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.
Pisani, A., Calabresi, P., Tozzi, A., D'Angelo, V., Bernardi, G., 1998. L-type Ca^{2+} channel blockers attenuate electrical changes and Ca^{2+} rise induced by oxygen/glucose deprivation in cortical neurons. Stroke 29, 196–202.
Pringle, A.K., Benham, C.D., Sim, L., Kennedy, J., Iannotti, F., Sundstorm, L.E., 1996. Selective N-type calcium channel antagonist omega conotoxin MVIIA is neuroprotective against hypoxic neurodegeneration in organotypic hippocampal-slice. Stroke 27, 2124–2130.
Rahbar-Roshandel, N., Razavi, L., Tavakoli-Far, B., 2007. Nimodipine protects PC12 cells against oxygen–glucose deprivation. IJPT 6, 51–54.
Rahbar-Roshandel, N., Razavi, L., Tavakoli-Far, B., Mahmoudian, M., 2008. Mebudipine and dibudipine protect PC12 cells against oxygen–glucose deprivation and glutamate induced cell death. Pathophysiology 15. doi:10.1016/g.pathophy.2008.09.002.
Rami, A., Krieglstein, J., 1994. Neuronal protective effects of calcium channel antagonists in cerebral ischemia. Life Sci. 55, 2105–2113.
Rothman, S.M., Olney, J.W., 1986. Glutamate and the pathophysiology of hypoxic-ischemic brain damage. Ann. Neuro. 19, 105–111.
Sanchez-Gomez, M.V., Alberdi, E., Ibarretxe, G., Torrre, I., Matute, C., 2003. Caspase-dependent and caspase-independent oligodendrocyte death mediated by AMPA and kainate receptors. J. Neurosci. 23, 9519–9528.
Sattler, R., Xiong, Z., Lu, W.Y., MacDonald, J.F., Tymianski, M., 2000. Distinct roles of synaptic and extrasynaptic NMDA receptors in excitotoxicity. J. Neurosci. 20, 22–33.
Siesjo, B.K., 1992a. Pathophysiology and treatment of focal cerebral ischaemia: part I. Pathophysiology. J. Neurosurg. 77, 337–354.
Siesjo, B.K., 1992b. Pathophysiology and treatment of focal cerebral ischaemia: part II. Mechanisms and drug treatment. J. Neurosurg. 77, 1247–1252.
Silver, I.A., Erecinska, M., 1990. Intracellular and extracellular changes of $[Ca^{2+}]$ in hypoxia and ischemia in rat brain *in vivo*. J. Gen. Physio. 95, 837–866.
Simeone, T.A., Sanchez, R.M., Rho, J.M., 2004. Molecular biology and ontogency of glutamate receptors in the mammalian central nervous system. J. Child Neurol. 19, 343–360.
Simon, R.P., Swan, J.H., Griffiths, T., Meldrum, B.S., 1984. Blocked of NMDA receptors may protect against ischemic damage in the brain. Science 226, 850–852.
Shibuta, S., Varathan, S., Varathan, V., Takemura, M., Yonehara, N., Mashimo, T., 2003. Effects of deep hypothermia on nitric oxide-induced cytotoxicity in primary cultures of cortical neurons. J. Neurosci. Res. 72, 613–621.
Warner, D.S., Sheng, H., Batinic'-Haberle, I., 2004. Oxidants, antioxidants and the ischemic brain. J. Exp. Biol. 207, 3221–3231.
Weaver, J., Porasuphatana, S., Tsai, P., Pou, S., Roman, L.J., Rosen, G.M., 2005. A comparative study of neuronal and inducible nitric oxide synthases: generation of nitric oxide, superoxide and hydrogen peroxide. Biochem. Biophys. Acta 1726, 302–308.
Whiteman, M., Armstrong, J.S., Cheung, N.S., Siau, J.L., Rose, P., Schantz, J.T., Jones, D.P., Halliwell, B., 2004. Peroxynitrite mediates calcium-dependent mitochondrial dysfunction and cell death via activation of calpains. FASEB J. 18, 1395–1397.
Yagami, T., Ueda, K., Sakaeda, T., Itoh, N., Sakaguchi, G., Okamura, N., Hori, Y., Fujimoto, M., 2004. Protective effects of a selective L-type voltage -sensitive calcium channel blocker, S-312-d, on neuronal cell death. Biochem. Pharmacol. 67, 1153–1165.
Yoshioka, A., Yamaya, Y., Saiki, S., Kanemoto, M., Hirose, G., Beesley, J., Pleasure, D., 2000. Non-*N*-methyl-D-aspartate glutamate receptors mediate oxygen–glucose deprivation-induced oligodendroglial injury. Brain Res. 854, 207–215.
Zhang, M., Ning, G.-M., Hong, D.-H., Yang, Y., Kutor, J., Zheng, X.-X., 2003. The influence of oxygen–glucose deprivation on nitric oxide and intracellular Ca^{2+} in cultured hippocampal neurons. Acta Biochem. Biophy. Sin. 35, 561–566.
Zhang, X.Q., Watanabe, Y., Ohnishi, M., Baba, T., Shibuya, T., 1993. Comparative studies on the inhibitory effects of calcium antagonists on cytosolic Ca^{2+} levels increased by high-potassium or glutamate in cultured rat cerebellar granule cells. Jpn. J. Pharmacol. 62, 411–414.
Zhang, Y.F., Fan, X.J., Li, X., Peng, L.L., Wang, G.H., Ke, K.F., Jiang, Z.L., 2008. Ginsenoside Rg1 protects neurons from hypoxic-ischemic injury possibly by inhibiting Ca^{2+} influx through NMDA receptors and L-type voltage-dependent Ca^{2+} channels. Eur. J. Pharmacol. 586, 90–99.