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Protective Effect of ^{25}Mg-Porphyrin-Fullerene Nanoparticles on Oxygen-Glucose Deprivation/Reperfusion Injury in PC12 Cells

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Abstract- We investigated the effects of ^{25}Mg-Porphyrin-Fullerene nanoparticles, (^{25}MgPMC16) smart ferroporphyrin nanoparticles, on PC12 cells exposed to oxygen-glucose deprivation/reperfusion. In order to explore its effect on cells under oxygen-glucose deprivation conditions, the cultures were pretreated with ^{25}MgPMC16 24 hours prior to oxygen-glucose deprivation/reperfusion. To initiate the oxygen-glucose deprivation/reperfusion, the cell culture medium was replaced with a 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, 60 and 120 min. Cell viability was assessed by MTT assay. Exposure of PC12 cells to 30, 60 and 120 min oxygen-glucose deprivation significantly decreased the cell viability. Pretreatment of the cultures with ^{25}MgPMC16 significantly increased cell viability in a concentration-dependent manner. Pretreatment, the cultures with MK-801 (10 µM), a non-competitive NMDA antagonist, has attenuated the cell death after 30 min oxygen-glucose deprivation. We concluded that ^{25}MgPMC16 could protect PC12 cells against oxygen-glucose deprivation/reperfusion-induced cell injury in a concentration-dependent manner. That could be due to the effect of ^{25}MgPMC16 on ATP synthesis and the antioxidant effects of its components.

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Introduction

A continuous blood flow to the brain supplies its need to oxygen and glucose. During the brain ischemia, an interruption in oxygen and glucose delivery causes a rapid reduction in energy available and thus, decreased ATP concentration. Therefore, it causes accumulation of the extracellular glutamate. Elevation in the extracellular glutamate levels induces a complex sequence of events that ultimately leads cell death (1,2).

Glutamate is the dominant excitatory neurotransmitter in the mammalian central nervous system and is involved in important neurophysiological functions including cognition, memory and learning (3,4). The healthy adult brain has the ability to clear extracellular glutamate by cellular glutamate uptake systems. During the brain ischemia, an impairment of glutamate uptake causes glutamate efflux to the extracellular space. The abnormally release of glutamate can cause neuronal cell death by a pathological process known as glutamate excitotoxicity (5-8). In physiological conditions, magnesium is a noncompetitive inhibitor of the NMDA receptors. Excessive activation of AMPA/kainate receptors can cause membrane depolarization, followed by expelling Mg^{2+}block of NMDA receptor, allows a large influx of Ca^{2+}through NMDA-gated channels (8-11).

Calcium is essential intracellular ion for proper function of neuronal cells. Several studies indicate that calcium could stimulate enzymes in mitochondria to increase the ATP synthesis. On the other hand, mitochondria have a pivotal role in the regulation of neuronal calcium signaling, neuronal calcium homeostasis, and calcium-dependent exocytosis. At high intracellular Ca^{2+}levels mitochondria cease respiration and evoking the generation of reactive oxygen species

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(1,7,12,13). However, the high levels of neuronal cytoplasmic calcium can activate several hydrolytic enzymes such as lipases, proteases, endonucleases. The stimulation of intracellular processes will increase the oxygen demand that further aggravates the hypoxia. All these events result in apoptotic or necrotic cell death (1,14).

Glutamate excitotoxicity and mitochondrial dysfunction have been linked to many human disease states such as stroke and chronic neurodegenerative disorders like Parkinson and Alzheimer (15-17). Therefore, prevention of the consequences of ischemic cell damage may provide effective therapeutic strategies. Recently, a porphyrin-attached fullerene-C60 nanoparticle of magnetic magnesium (^{25}Mg PMC16) which possesses cationite properties has been designed for the correction of ATP synthesis in oxygen-depleted cells (18,19). This low toxic nanoparticle with amphiphilic membranotropic properties is the iron containing porphyrin monoadduct of a classical Buckminsterfullerene, buckminsterfullerene (C60)-2-(butadiene-1-yl)-tetra (o-γ-aminobutyryl-o-phthalyl) ferroporphyrin, named ‘‘Porphylleren-MC16’’ or, in brief, PMC16 (18). This smart nanoparticle has unique physical and chemical properties. The non-allergic, anti-inflammation and high biocompatibility of its components make PMC16 suitable for pharmacological studies (18). The ^{25}Mg isotopes that released by this nanoparticle activate both substrate and oxidative phosphorylation pathways and stimulate the production of ATP in oxygen-depleted cells (18,20). Furthermore, the protective effects of magnesium salts against brain ischemia have been shown in several studies. These studies indicate that magnesium could attenuate neuronal death by a various mechanism including inhibition of NO production, inhibition of NMDA glutamate receptors and the regulation of Ca^{2+}accumulation (20-23).

In this study the protective effect of ^{25}Mg PMC16 on PC12 cells, a rat pheochromocytoma cell line, against oxygen-glucose deprivation/reperfusion induced neurotoxicity has been studied.

Materials and Methods

Chemicals and reagents

^{25}MgPMC16 was received from the Semenov institute (Russian Academy of Sciences) and dissolved in 1 mM Na-EDTA and 15 mM Na phosphate buffer at a concentration of 1 mg/ml. The solution was further diluted with RPMI to obtain the desired concentrations. The highest concentration of EDTA in each well of the plates was lower than 0.1% to prevent the significant cytotoxic effects on the PC12 cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), MK-801 were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Cell culture media RPMI-1640 and Dulbecco’s modified Eagle’s medium (DMEM), Fetal Bovine Serum (FBS), Horse Serum (HS), and penicillin-streptomycin were purchased from GIBCO BRL.

Cell line

PC12 cells were obtained from the Pasteur institute of Iran (Tehran, Iran) and were grown in RPMI-1640, supplemented with 10% FBS (heat-inactivated), 5% HS (heat-inactivated), 100 IU/ml penicillin and 100 µg/ml streptomycin, in a humidified incubator aerated with 5% CO_2 in air at 37°C. The cells were subcultured twice a week by gentle scraping and cultured at a density of 6-8×10^5cells/ml in 96-wells plates. Cells were used for experiments 24 h after seeding.

Drug administration and oxygen-glucose deprivation

Cells were treated 24 h before oxygen-glucose deprivation with ^{25}Mg PMC16 at the concentrations of 10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml. These concentrations were chosen based on the results of preliminary experiments. To investigate the effects of glutamate receptor inhibitors on oxygen-glucose deprivation-induced cell death, MK801, a non-competitive antagonist of the NMDA receptor, was added to the medium 24 h before the oxygen-glucose deprivation.

The model of oxygen-glucose deprivation was performed as described previously (24). Briefly, the culture medium was replaced with glucose/glutamine-free DMEM and was exposed to hypoxia for 30, 60 and 120 minutes 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.

Analysis of cell viability

Cell viability was monitored using the colorimetric MTT assay as previously described (25). Cells were incubated with 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, was dissolved in 100 µl dimethylsulfoxide (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 the control normoxic plates determined by MTT reduction.

Statistical analysis

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

Results

The effects of ^{25}MgPMC16 on oxygen-glucose deprivation/24h reperfusion-induced cell injury on PC12 cell line

We examined the effects of ^{25}Mg PMC16 on 30, 60 and 120 min oxygen-glucose deprivation/24h reperfusion induced cell injury in PC12 cells. Cell cultures were pretreated with ^{25}Mg PMC16, 24 hours before exposure to 30, 60 and 120 min of oxygen-glucose deprivation.

Pretreatment of cell cultures with ^{25}Mg PMC16 (10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml) 24 h before exposure to 30,60 and 120 min oxygen-glucose deprivation significantly increased the percentage of viable cells in a concentration-dependent manner (Figures 1-3).

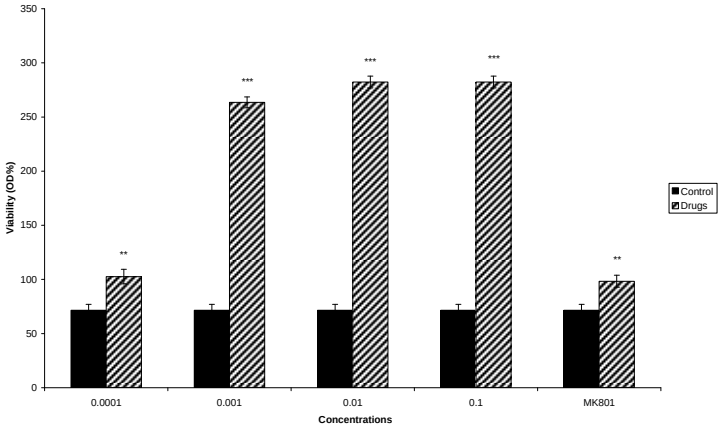

Figure 1. The effects of ^{25}Mg PMC16 (10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml) and MK-801 (10 μM) during 30 min oxygen-glucose deprivation/24 h reperfusion- induced cell injury on PC12 cell culture. The dashed bar shows the drug group and the black bar shows the control group. Optical density 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%). $^{**}P< 0.005$, $^{***}P< 0.0005$

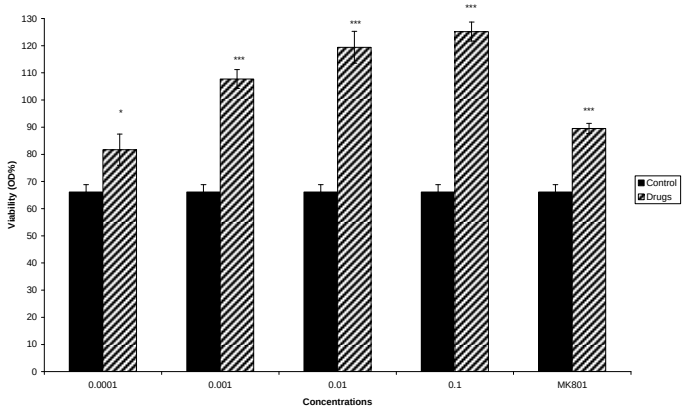

Figure 2. The effects of ^{25}Mg PMC16 (10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml) and MK-801 (10 μM) during 60 min oxygen-glucose deprivation/24 h reperfusion- induced cell injury on PC12 cell culture. The dashed bar shows the drug group and the black bar shows the control group. Optical density 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%). $^{*}P<0.05$, $^{***}P<0.0005$

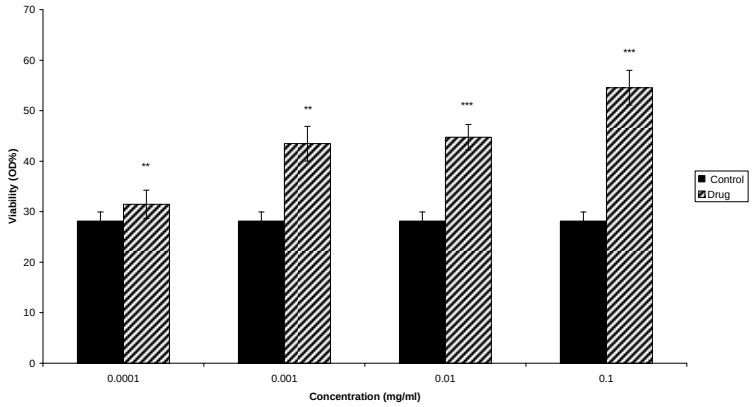

Figure 3. The effects of ^{25}Mg PMC16 (10^{-4}, 10^{-3}, 10^{-2}, and 10^{-1} mg/ml) during 120 min oxygen-glucose deprivation/24 h reperfusion- induced cell injury on PC12 cell culture. The dashed bar shows the drug group and the black bar shows the control group. Optical density at 570 nm corresponds 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*<0.005, ****P*<0.0005

The effects of MK-801 on oxygen-glucose deprivation/24h reperfusion-induced cell injury on PC12 cell line

MK-801, a non-competitive NMDA receptor antagonist (10 μM), significantly increased cell viability in 30 min oxygen-glucose deprivation (Figure 1). MK-801(10 μM) partially increased cell viability in 60 min oxygen-glucose deprivation (Figure 2).

So it seems that activation of NMDA receptors is the major cause of cellular damage during 30 or 60 minutes of OGD/R. It also shows that the pathway followed by OGD/R is the same pathway as excitotoxicity (that has been proven in various studies) (9,26).

During 120 minutes OGD/R, MK801 did not have any significant inhibitory effect on cell death. It shows the role of glutamate-induced cell death just at the early stage of excitotoxicity cascade.

Discussion

In the current study, we examined whether ^{25}MgPMC16 can protect PC12 cells against oxygen-glucose deprivation. PC12 cells are a rat Pheochromocytoma cell line represents as a suitable model of neurons (26,27). We used oxygen glucose deprivation as a well-characterized *in vitro* model for the induction of neuronal cell injury. Our results indicated that ^{25}MgPMC16 could effectively protect the PC12 cells against cell death induced by oxygen-glucose deprivation/reperfusion in all three time schedules. We observed that when cells exposed to 30,60 and 120 minutes oxygen-glucose deprivation/reperfusion the drug dramatically suppressed the PC12 cell death in a concentration-dependent manner. MK-801 an antagonist of glutamate NMDA receptors significantly attenuated 30 minutes oxygen-glucose deprivation/reperfusion induced neurotoxicity but partially increased cell viability in 60 min oxygen-glucose deprivation.

Due to cationic properties of ^{25}MgPMC16, this smart nanoparticle has the ability to deliver its magnetic magnesium only in an acidosis condition which is a natural consequence of any type of hypoxia (18). As mentioned in the introduction, $^{25}Mg^{2+}$ activates both pathways of ATP synthesis (18,28). Magnesium has a catalytic effect and possible structural role in creatine kinase and Mg-containing enzymes of mitochondrial respiratory chain (19). Beside the remarkable improvement in ATP production and regulation of Ca^{2+} accumulation, magnesium is involved in the facilitation of Na^+/K^+ ATPase function, ion gradients stabilization, protein synthesis and maintaining membrane integrity (23,29).

The neuroprotective effect of magnesium has been shown in many experimental studies. It has been shown that magnesium sulfate reduces cerebral injury in transient cerebral ischemia in rats (30). Another study suggested that treatment with $MgCl_2$ is effective in the attenuating of the neurological damage in experimental rat brain injury (29). Also, it has been shown that the neuroprotective effect of magnesium is due to its inhibition of NO production in fetal hippocampal slices after oxygen-glucose deprivation (21). In addition, previous studies indicated that under ischemic conditions, magnesium could attenuate the neuronal cell

death by inhibition of neurotransmitter release and anoxia-induced depolarization (22,30,31). However, Muir *et al*., in a randomized controlled trial, examined the effect of intravenous magnesium sulfate in patients with acute stroke. The results indicated that magnesium given within 12 h of acute stroke did not reduce the death or disability significantly (32). According to our results, the protective effect of ^{25}MgPMC16 on PC12 cell death during oxygen-glucose/deprivation might be partially due to the release of its $25Mg^{2+}$ in acidosis condition of ischemia.

In previous studies in hypoxic cardiopathies, ^{25}MgPMC16 protected myocardium cells of serious damage of hypoxia. ^{25}Mg released by these nanoparticles stimulated the production of ATP in oxygen-depleted cells (18,33,34). In another study, it has been shown that ^{25}MgPMC16 could protect the rat lymphocytes against chemical hypoxia induced by energy metabolism inhibitors (19). Recently, in experimental diabetic neuropathy, ^{25}MgPMC16 protected Dorsal Rat Ganglion (DRG) neurons from cell injury and also caused significant changes in oxidative stress biomarkers in comparison to diabetic neuropathy control groups (20). Measurements of ATP production and ADP/ATP in the presence of ^{25}MgPMC16 indicated that this nanoparticle increases the total rate of ATP synthesis via substrate phosphorylation and oxidative phosphorylation in mitochondria; therefore ^{25}MgPMC16 could protect the heart muscle cells and DRG neurons from cell death (18,20).

According to our results, it could be suggested that at the early stage of excitotoxicity, ^{25}MgPMC16 could decrease the extracellular glutamate by over production of ATP synthesis via the improvement of ATP-dependent glutamate transporters function. On the other hand, the excessive cytoplasmic Ca^{2+} levels, due to Ca^{2+} influx, is sequestered into the mitochondria and decreases the electrochemical gradient, followed by a decline in ATP synthesis. Concurrently, mitochondria should extrude excessive calcium from the membrane which is an ATP dependent process (7,35). Primary inhibition of the mitochondrial respiratory chain indirectly induced NMDA receptor stimulation, which is termed as secondary excitotoxicity (11). It is likely that ^{25}MgPMC16 compensate ATP deficiency and supply energy required to maintain ionic gradient so that Ca^{2+} pump can push out extra Ca^{2+} and might prevent the occurrence of the secondary excitotoxicity.

Oxidative stress is a contributing factor in excitotoxicity through the generation of oxygen radicals and plays a critical role in the progression of neurodegenerative diseases (36-39). It has been demonstrated that fullerene derivatives are excellent antioxidant agents and characterize as radical sponges against excitotoxic cell death (40-43). They could decrease the toxicity of free radical on the neuronal tissue (42). On the other hand, Mg^{2+} competes with calcium ions for binding sites on mitochondrial membrane, that resulting in a reduction of Ca^{2+}-induced mitochondrial reactive oxygen species generation (44,45). Taking together, it could be suggested that, ^{25}MgPMC16, with Mg^{2+} and fullerene C60 structure, could attenuate cell injury-induced oxidative stress.

According to our results, ^{25}MgPMC16 could significantly protect cells against cell death, even during the long periods of oxygen-glucose deprivation/reperfusion (120 min). It seems that the long-lasting effect of PMC16 with magnetic magnesium is due to its unique structure, including magnesium, porphyrin, and C60 fullerene. Porphyrin domain of PMC16 has high affinity to receptors located in the external membrane of the mitochondria, and C60 fullerene, selectivity accumulate inside mammalian mitochondria (18, 20).

In conclusion, ^{25}MgPMC16 nanoparticles could affect in the several parts of excitotoxicity cascade thereby could protect cells from cell death. dPossible mechanism of the protective effect of ^{25}MgPMC16 could be summarized as target delivery of magnesium, positive role in the improvement of ATP production and antioxidant properties of its components. These findings may be useful for the development of a new generation of drugs against stroke and chronic neurodegeneration disorders.

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