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Neuronal Cytotoxicity of Oxygen Radical in Newborn Mouse Forebrain Culture

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ABSTRACT : *The cytotoxic effects of hydrogen peroxide and neuroprotective effects of a variety of agents were investigated in newborn mouse forebrain tissue culture. In our experiments, oxygen radical was generated enzymatically by glucose oxidase and the values were expressed as a percentage of number of living cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cytotoxicity of oxygen radicals was prevented by catalase and (N,N,N',N'-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), but N-tetra-α-butyl-phenylnitrone (PBN), and deferoxamine (DFX), failed to show protective effects against oxygen radicals. Antagonists of the N-methyl-D-aspartate (NMDA) receptor, D-2-amino-5-phosphonovaleric acid (APV), 7-chlorokynurenic acid (CKA), and MK801 (a non-competitive NMDA antagonist) were also not effective in blocking neurotoxicity induced by glucose oxidase generated oxygen radicals.*

Key Words : *Mouse forebrain, Oxygen radical, Catalase, TPEN*

I. INTRODUCTION

Tissue damage as a result of oxidant production by phagocytes occurs at several levels, and results from the generation of various toxic oxygen species. Many enzymes in cytoplasm can generate hydroxyl radical which has various possible targets, such as membranes and DNA. Hydrogen peroxide contributed by several enzymes causes breakdown of ATP, ultimately it was contributed to hypoxanthine and xanthine, or glucose, which is substrates for xanthine oxidase or carbohydrate compounds. The product is superoxide or hydrogen peroxide, which can reduce Fe^{3+} to Fe^{2+}, after that fenton reaction produces hydroxyl radical. Several common neurological diseases are suspected to involve a combination of interacting excitatory and oxidative processes. In particular, formation of free radicals resulting from oxidase activation is responsible for peroxidative degradation of lipid membranes and other destructive events (Chan and Fishman, 1978; Lundgren *et al.*, 1991; Weiss and Haber, 1984; Rubin and Faber, 1984). Generally, free radical is produced in normal metabolism, while this free radical oxidizes especially the lipid layers of brain cell membranes.

Recently many investigators have suggested that the reason for brain dysfunction, spinal cord injury (Hall and Braughler, 1986; Saunders *et al.*, 1987) and stroke (Amamoto *et al.*, 1983; Hall and Braughler, 1989) is oxidative stress. Because cytotoxicity is mediated by oxygen radical, it can lead to neuronal death. Ischemia (Harken *et al.*, 1988), Parkinson's disease (Slivka and Cohen, 1985) and Huntington's disease (Dexter *et al.*, 1989) have been reported to be caused by iron mediated radical production. Many recent studies have reported that either oxygen radical scavenger or iron chelator improves neuronal cell survival in culture systems. The oxidative reaction of lipid layer in cell membrane is accelerated by iron metal ion. Brain tissue is especially susceptible to oxidative damage, because it is enriched in highly unsaturated fatty acids particularly in certain regions that contain high iron. It is known that several oxygen scavengers can protect against oxygen radical in cell culture. Recently, antioxidant therapy has been suggested for several neural diseases (Rice-Evans and Diplock, 1993).

This study uses a radical generated GO system as an in vitro model of disease caused by oxidative damage. The goal of this study is to examine the potential

protective effects of oxygen radical scavengers and NMDA receptor antagonists in a newborn mouse brain tissue culture system.

II. MATERIALS AND METHODS

1. Cell Culture

Newborn mouse brain was cultured in 96 multiwell plates as described previously (Michikawa *et al.*, 1994). Primary cultures of forebrain were prepared from newborn mice. The brain tissues were washed to remove blood and debris. Under a microscope, tissue was dissected with a razor and then incubated in 0.25% trypsin and 20 μg/ml DNase in Dulbecco's phosphate-buffered saline (PBS) without calcium and magnesium for 30 minutes at 37°C, 5% CO_2/95% air. Following the incubation, trypsin and DNase were removed by centrifugation at 800 rpm for 5 minutes. The pellet was washed with medium consisting of Eagle's minimum essential medium (MEM), 10% fetal bovine serum (FBS), 5 mg/ml D-glucose, and 20 μg/ml gentamicin and centrifuged again. The precipitated pellet was gently resuspended with Pasteur pipette several times to make single cells. The single cells were divided in 96 multiwells coated with 10 μg/ml poly-L-lysine. A cell density was at most 1×10^5 cells/well. All multiwells containing newborn forebrain cells were incubated at 37°C, 5% CO_2/95% air until the experimental day. Cells were used for these experiments after 10-14 days in culture.

2. Chemicals

Glucose oxidase (GO) (type x from Aspergillus niger, G8135), deferoxamine (DFX), N,N,N',N',-tetrakis (2-pyridylmethyl) ethylenediamine(TPEN), catalase (from bovine liver, C40), D-2-amino-5-phosphonovaleric acid (APV) (A5282), poly-L-lysine (MW > 300,000), and 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemicals (St. Louis, Mo). 7-chlorokynurenic acid (CKA) was obtained from Tocris Neuramin (UK), and MK801 was provided by Merck Sharp & Dohme Research Lab (Rahway, NJ). N-tetra-α-butyl-phenylnitrone (PBN) was obtained from Aldrich Chemicals (Milwaukee, WI).

3. Exposure to Oxygen Radicals

Oxygen radical formation was initiated by glucose oxidase (GO). The cultured cells were washed three times with MEM containing 0.5% glucose (exposure medium) to remove serum. In this experiment, cells were preincubated in the exposure medium with oxygen radical scavengers for 1 hr at 37°C, 5% CO_2/95% air. After the preincubation with oxygen radical scavengers, the cells were gently washed three times with the same medium and reacted with the oxygen radical generating system by adding a variable concentration of GO to each well. Incubation time of cells was 1-4 hrs at 37°C, 5% CO_2/95% air. When NMDA receptor antagonists (APV, CKA, and MK801) were used, they were added simultaneously with the oxygen radical generating system. In the next step, cells were used for MTT cytotoxicity assay (Mosmann, 1983; Francois and Lang, 1986).

4. MTT Cytotoxicity Assay

This assay was carried out as described in a previous paper (Michikawa *et al.*, 1994). MTT cytotoxicity assay was performed after appropriate incubation periods for protective effects of radical scavengers and NMDA receptor antagonists. 10 μl of MTT stock solution (5 mg/ml) was added to a final volume, 100 μl incubated medium in each well. The MTT addition mixture was incubated for 4 hrs at 37°C, 5% CO_2/95% air. After incubation, MTT solution was removed from the reaction medium. The acidic isopropanol (200 μl) was added to each well. The 96 multiwell was then measured on a Dynatech Microelisa reader at a wavelength of 570 nm. The neuroprotective effects against oxygen radicals were analyzed statistically single factor ANOVA analysis with $p < 0.01$.

III. RESULTS

1. Cytotoxicity of Glucose Oxidase-Induced Oxygen Radical

The first two experiments were studied the effects of glucose oxidase concentration and incubation time on cell survival, after newborn

mouse brain culture for two weeks. To evaluate the dose response relationship of GO generated oxygen radical cytotoxicity on newborn mouse brain, cells in 96 multiwells were exposed to concentrations of 1, 5, 10, 20, and 40 mU/ml GO for 4 hrs, and then processed for the MTT assay.

The number of living cells varied inversely with GO concentration. At 1 mU/ml of GO, the number of living cells was about 96% of all the unexposed cells. At 10 mU/ml of GO, 75% of the cells was survived. At 20 mU/ml, and 40 mU/ml, 45% and 30% were survived respectively. At various concentrations, total cell population survived against oxygen radical induced cytotoxicity is shown in Fig. 1A. Based on these resutls, a concentration of 20 mU/ml GO was used for subsequent studies of cytotoxicity in newborn mouse brain cultures. The effects of glucose oxidase incubation time on cell survival are shown in Fig. 1B. The number of cells which were stained with MTT solutions was 87% after incubation of 30 minutes, 84% after 1 hr, 61% after 2 hrs, 54% after 3 hrs, and 44% after 4 hrs.

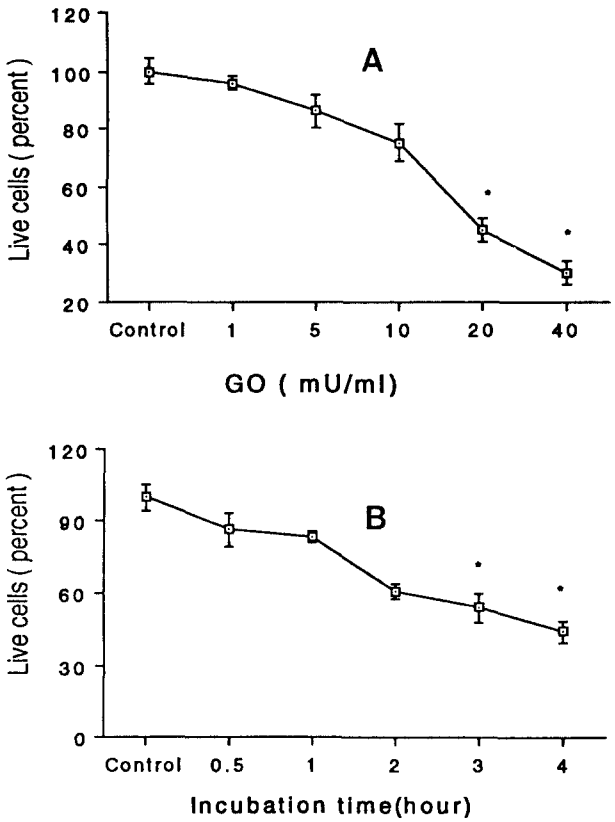

Fig. 1-A. A dose response relationship of glucose oxidase (GO) concentration on GO mediated neurotoxicity as measured by MTT assay in newborn mouse forebrain. Reduction in number of living cells following treatment with glucose oxidase (GO) for 4 hrs incubation at various concentrations (1, 5, 10, 20, and 40 mU/ml). The values are expressed as a percentage of control value. The results indicated the mean±SEM for 4-6 experiments. *P < 0.01, significantly different from the control. Single factor ANOVA analysis.

Fig. 1-B. A time dependency of GO mediated oxygen radical neurotoxicity as measured by MTT assay in newborn mouse brain. Cultures were exposed to 20 mU/ml GO for 0.5, 1, 2, 3, and 4 hours. The values are expressed as percentage of number of living cells by MTT assay following exposure to GO (20 mU/ml). The results indicated the mean±SEM for 4-6 experiments. *P < 0.01, significantly different from the control. Single factor ANOVA analysis.

2. The Effects of Radical Scavengers on GO Mediated Cytotoxicity

Various agents were tested for their ability to protect against GO mediated cytotoxicity in this system. An incubation of 4 hrs with 20 mU/ml glucose oxidase was done for this study.

Catalase, a radical scavenger, an enzyme which removes hydrogen peroxide, protected the cells against GO generated oxygen radical. At a concentration of 5 μg/ml catalase, approximately 86% of the cells was survived, this protective effect was statistically significant ($p < 0.01$). At 20 μg/ml catalase, over 88% of the cells was remained alive, compared with control (Fig. 2). To investigate the

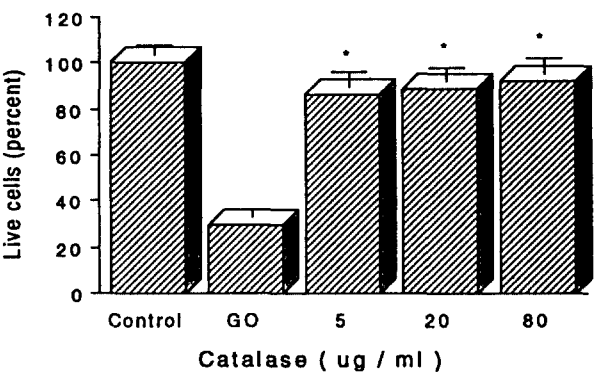

Fig. 2. A dose response relationship of catalase for its neuroprotective effect against oxygen radical. The newborn mouse brain was preincubated with catalase at concentrations of 5, 20, and 80 μg/ml for 1 hr, washed, and exposed to oxygen radicals generated by 20 mU/ml GO for 4 hrs. Cell viability was determined by MTT assay. The results are mean±SEM of 4-6 experiments. *P < 0.01, significantly different from the cultures exposed to GO alone. Single factor ANOVA analysis.

involvement of iron in the GO generated oxygen radical system, cytotoxicity mediated by GO in newborn mouse brain cultures was assessed after the addition of the different two metal chelators, TPEN, and DFX. One of them, TPEN complexes with two positively charged metal ions (Arslan *et al.*, 1985). They were added to the culture in the multiwell for 1 hr before it was exposed to GO. Then the cultures were washed two times with culture medium, and exposed to GO according to the standard procedure. At a concentration of 10 μM TPEN, the number of living cell was higher than that of only treated GO, indicating that TPEN is very effective in blocking the neurotoxicity by GO generated oxygen radicals (Fig. 3). TPEN had definitive protective effects over at a concentration of 10 μM. Although the concentration of TPEN was low, the protective effects against oxygen radical were statistically significant $p < 0.01$ (Fig. 3A). DFX did not show any protect effect at concentrations of 100, 200, and 400 μM (Fig. 3B). PBN was also shown same tendency in protective effect against GO generated oxygen radicals (Fig. 4).

Effects of NMDA receptor antagonists were assessed by exposure of APV, CKA, and MK801, and were slightly effective in blocking oxygen radical cytotoxicity, but effects were not significant even at $p < 0.05$ (Fig. 5).

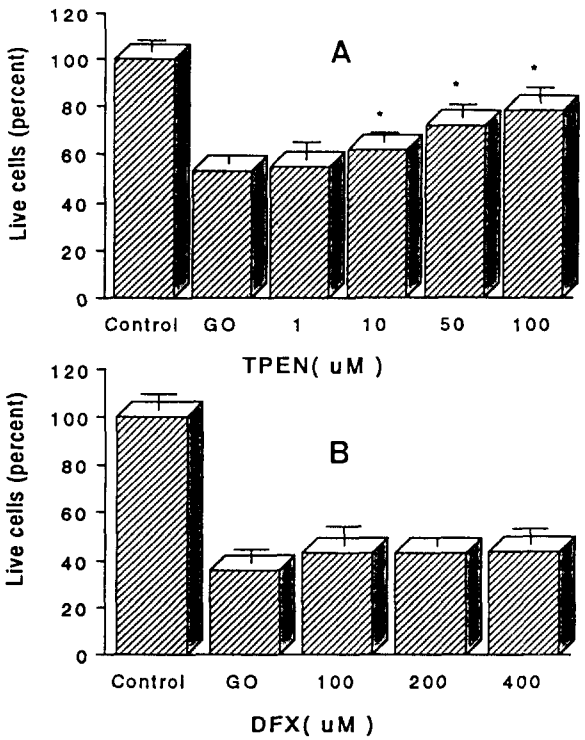

Fig. 3. A dose response relationship of metal chelators, tetrakis (2-pyridylmethyl) ethylenediamine (TPEN), and deferoxamine (DFX) for their neuroprotective effects against oxygen radicals. The newborn mouse brain was preincubated with reagents for 1 hr, washed, and then exposed to oxygen radicals generated by 20 mU/ml GO for 4 hrs. The newborn mouse brain was preincubated with TPEN at the concentrations of 1, 10, 50, and 100 μM(A), and with DFX at concentrations of 100, 200, and 400 μM(B). TPEN at the concentration of 10μM blocked the oxidant mediated neurotoxicity but DFX did not show any neuroprotective effect against oxygen radicals. Cell viability was determined by MTT assay. The results are mean±SEM of 4-6 experiments. *P < 0.01, Significantly different from the cultures exposed to GO alone. Single factor ANOVA analysis.

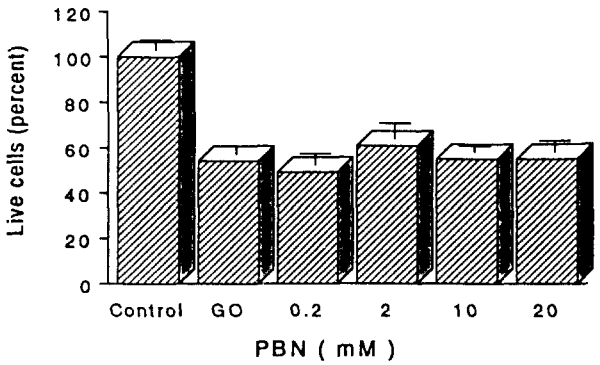

Fig. 4. Effects of N-tetra-α-butylphenylnitrone (PBN) on GO mediated cytotoxicity. The newborn mouse brain was preincubated with various concentrations of N-tetra-α-butylphenylnitrone and oxygen radicals generated by 20 mU/ml GO for 4 hrs. PBN did not show any neuroprotective effects against oxygen radicals. The results are mean±SEM of 4-6 experiments.

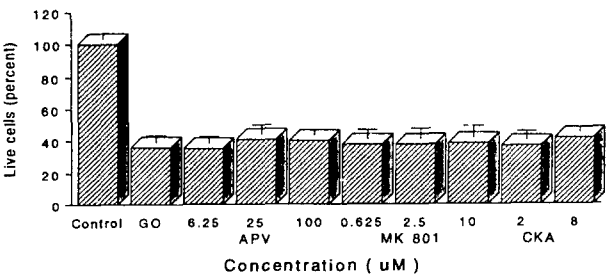

Fig. 5. Effects of NMDA receptor antagonists, APV (2-amino-5-phosphovaleric acid), MK801, and CKA (7-chlorokynurenic acid), on GO mediated cytotoxicity. The newborn mouse brain was preincubated with one of the NMDA receptor antagonist reagents and oxygen radicals generated by 20 mU/ml GO for 4 hrs. Protective effects were not statistically significant. The results are mean± SEM of 4-6 experiments. *P < 0.05, significantly different from the cultures exposed to GO alone. Single factor ANOVA analysis.

IV. DISCUSSION

Two theories of neuronal cell death have been suggested. One of them is radical reaction with the cell membrane, another is NMDA agonist, or a combination of both interactions. It is known that the cell membrane is a primary site of oxygen radical mediated injury, owing to its content of peroxidizable unsaturated lipids and proteins and to its physical proximity both of intracellular and extracellular radical sources. NMDA agonists can contribute to lipid layer oxidation by radical. NMDA agonists are especially potent in the stimulation of nitric oxide synthetase and nitric oxide can interact with superoxide to form the intensely oxidant nitroperoxyl radical.

Glucose oxidase acts on glucose in culture medium and produces hydrogen peroxide directly at a slow continuous rate. Owing to this hydrogen peroxide radical, the cascade reaction will go on with iron. Finally, the living cells were killed mostly in 4 hrs incubation. Under these conditions, the number of dead cells was averaged 56%.

Our results in a GO genarated agents, radical system showed that catalase and TPEN were only significantly blocked hydrogen peroxide radical mediated cytotoxicity. Catalase, an enzyme which removes hydrogen peroxide, has previously been shown to block oxidant-mediated neurotoxicity in other model systems investigated such as cerebellar neuron culture (Dykens *et al.*, 1987), hippocampal slices (Pellegrini-Giampietro *et al.*, 1988), oligodendrocyte cultures (Kim and Kim, 1991), and spinal cord cultures (Michikawa *et al.*, 1994). TPEN significantly blocked cytotoxic effects of oxygen radical although it was added only in trace concentration. Our opinion is that TPEN acts by chelating another bivalent cationic ions e.g. Ca^{2+} which are known to play a role in neurotoxicity induced by excitotoxic amino acids (Choi, 1987), not iron. Another thinking is that the difference in action between these chelators in blocking oxygen radical neurotoxicity is reflected by their chemical structure and biological activity. DFX is a hydrophilic membrane iron chelator and is not membrane permeable, while TPEN is permeable through the cell membrane and can chelate heavy metals such ferric ion and another two positive charged ions.

Deferoxamine failed to demonstrate any protective effects on the newborn mouse brain culture. This could be possible because there is either no iron or only a small amount of iron in the culture. Another possibility might be that iron chelator can not penetrate into cell membranes (Lioyd *et al.*, 1991). We have tried to induce neurotoxicity with 100 μM/ml Fe^{3+} and 2 mM/ml vitamin C without GO, and obtained 30% cell death at least 16 hrs of co-incubation (data not shown). Toxicity did not occur after 4 hrs incubation with iron alone at the same concentration. Normally neural cells contain only a trace amount of iron. We assume that the amount of iron in neural cells is stable even though the experimental incubations are long because the neural cells can not proliferate.

NMDA receptor antagonists were tested for neuroprotection. APV is a competitive NMDA antagonist; CKA, an antagonist at the glycine site associated with the NMDA receptor; and MK801, a non-competitive antagonist of NMDA receptor-ion channel complex. We recognized slight blocking effects in high concentration of MK801, CKA and APV, even though the cultures were used the high doses and over two weeks culture.

From these results and other observations, it is reasonable to suggest that hydrogen peroxide generated by GO is toxic agent for newborn mouse brain in this system. Why the metal chelator, TPEN should only protect against cytotoxicity in this system when deferoxamine did not, so it requires further study. We plan to investigate the mechanism of the interaction between lipid layer components, iron, and iron chelator in and out side cell membranes.

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