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Original Research Article

Neuroprotective Effect of Insulin-like Growth Factor-II on 1-Methyl-4-Phenyl Pyridinium-Induced Oxidative Damage in Cortical Neuronal Cells

Wei Dong[1], Lin Hu[2] and Xiao-Wei Xu[3]*
[1]Intensive Care Unit, The First People's Hospital of Jining, Jining 272011, [2]Intensive Care Unit, The People's Hospital of Zoucheng, Zoucheng, 273500, [3]Department of Emergency Medicine, The People's Hospital of Yanzhou, Jining 272100, China

For correspondence:* ***Email:* *meili77072@gmail.com;* ***Tel/Fax:*** *0086-537-3412259*

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Abstract

Purpose: *To evaluate the receptor-mediated neuroprotective effect of insulin-like growth factor-II (IGF-II) on 1-methyl-4-phenyl pyridinium (MPP)-induced oxidative damage in adult cortical neuronal cultures.*
Methods: *Adult rats were randomly divided into 5 groups. Cortical neurons were prepared from rats. The cells were exposed to 10 µM of MPP (group 1, G1); MPP + 100 ng/mL of IGF-II (group 2, G2); MPP + IGF in the presence of 20 ng/µL IGF-I analogue (group 3; G3); 5 ng/µL anti-IGF-IIR (group 4; G4); or MPP + IGF II + IGF inhibitor (group 5; G5). The level of reactive oxygen species (ROS), levels of oxidative stress markers, antioxidant enzymes, mitochondrial functional markers were analyzed in the MPP-treated neuronal cells (with or without treatment with IGF-II).*
Results: *The results demonstrate that IGF-II treatment protects MPP-induced toxicity by decreasing ROS production (58.33 %; $p < 0.001$), AChE levels (50 %), and maintaining the innate antioxidants to near normal levels. The study on oxidative functional markers showed that IGF-II significantly decreased the MPP-induced elevated levels and mitochondrial markers (TBARS, 40 %, LOOH-39.28 %) to near normal levels. Further analysis using inhibitors of IGF-IR (IGF-I analogue) and IGF-IIR (anti-IGF-IIR) showed that involvement of IGF-IIR might have greatly contributed to the neuroprotective effect of IGF-II.*
Conclusion: *IGF-II receptors play a significant role in the neuroprotective mechanism of IGF-II by acting as an antioxidant, thereby reducing the neuro-degeneration induced by oxidative insults. This indicates that IGF-II receptors are a potential target for the treatment of diseases related to imbalance in redox homeostasis.*

Keywords: *Insulin-like growth factor-II, Neuronal cells, 1-Methyl-4-phenyl pyridinium, Mitochondrial markers, Oxidative stress, Neuroprotection, Antioxidant*

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INTRODUCTION

MPP is a highly toxic molecule which acts by interfering with oxidative phosphorylation in mitochondria, causing depletion of ATP and cell death [1]. It also inhibits the synthesis of catecholamines, reduces levels of dopamine and cardiac norepinephrine, and inactivates tyrosine hydroxylase. Recently, the use of neurotoxin 1-methyl-4-phenylpyridinium (MPP), has enabled scientists to gain an insight into neurodegenerative pathogenesis experimentally. MPP has been reported to be a selective and potent inhibitor of complex I of the mitochondrial electron transport chain, leading to Parkinsonian symptoms in animal models [1,2].

Insulin-like growth factors are naturally occurring peptides with known pleiotropic effects as growth factors(3) The majority of its actions are mediated primarily by insulin receptors, IGF-I receptors (IGF-IRs) and, IGF-II/mannose-6-phosphate receptors (IGF-IIRs) [3]. Studies revealed that several IGF-binding proteins (IGFBPs) might enhance or abrogate the effects of IGF [4-6]. IGF-II, a 67 amino acid peptide is synthesized highly during embryonic development. However, in adults, there is a decrease in its synthesis, although IGF-II remains the most abundant insulin-like peptide in the adult brain, primarily expressed in the choroid plexus, the leptomeninges and the hypothalamus [7]. *In vitro* studies have shown the neuroprotective effects of IGFs (I and II) as antioxidants via the regulation of intracellular calcium levels and the restoration of mitochondrial membrane potential (MMP) [8,9]. Administration of low doses of IGF-II to 2-year old rats induced neuroprotective effects and decreased the levels of oxidative stress markers in the hippocampus and the cortex. These effects were attributed to IGF-II, as IGF-I plasma levels remained unchanged in these experimental animals [10].

Keeping in view the role of IGF-II receptors in neuroprotection, the present study was designed to elucidate the neuroprotective effect of IGF-II against the MPP-induced neurotoxicity using *in-vitro* culture of adult cortical neuronal cells isolated from rat brain.

EXPERIMENTAL

Preparation of cortical neuronal cultures

The animals used were maintained in accordance with the Chinese legislation guidelines and according to the Helsinki Humanity Research Declaration during the experiments and the euthanasia of the animals. All the protocols were approved by the Ethical Committee of Department of Neurology, Yantai Economic and Technology Development Area Hospital, Yantai 264006, China (approval ref no. YETDAHY/2014/01072-A-2014).

The cortical neurons were prepared from adult rats according to the method of Brewer [11]. The animals were decapitated and the cortex was subdivided into minute sections and then incubated in Hibernate ™-A/B27™ (Life Technologies, China) and papain for 30 min at 30 °C in a gentle-shaking water bath. The cells obtained from triturating of sections were suspended in OptiPrep gradient medium (Sigma Aldrich) and centrifuged at 800 ×g for 15 min. The fractions containing neurons were collected, suspended in Hibernate ™-A/B27 and centrifuged twice at 200 ×g for 2 min at 20 ^{o}C. The pellets were then resuspended in growth culture medium. For cell culture, 6- and 24-well plates were pre-coated with 100 µg/mL of poly-D-lysine.

For immunohistochemical studies, glass coverslips (12 mm diameter) were pretreated with polyethylenimine (1/500 v/v in ddH_2O) followed by 2.5 % FBS. Approximately 5 x 103 (6-well plates) or 1.25 x 10^4 (24-well plates) cells were seeded. The neurons were then incubated at 37 °C in 5 % CO_2, and after 1 h, the wells were rinsed twice with Neurobasal™-A and filled with growth culture medium. The media were replaced with fresh media (half of the initial volume) every 4 days. Cell growth was monitored for 12 days before commencing the experiments. Table 1 shows the treatment of cortical neuronal cells and determination of experimental groups (G1 to G5).

Determination of cell death and ROS measurement

As IGF-II is known for the activation of IGF receptors IGF-IR and IGF-IIR, we performed the receptor-inhibition study to reveal the involvement of IGF receptors during the IGF-II treatment against MPP. The neuronal cells were seeded in a 96-well tissue culture plate, at a density of approximately 1.25 x 10^4 cells/well, for 24 h the cells that were in serum-free media for 24 h were subjected to measurement of ROS production and cell death. Intracellular ROS generations were measured using a well-characterized probe, DCFH-DA. DCFH-DA is hydrolyzed by esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This non fluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by the action of cellular oxidants. The cells of experimental groups were also subjected to DCFH staining and the fluorescence was determined at 485 nm excitation and 520 nm emission, using a micro plate reader (FLUO star, BMG Lab technologies, NC, USA).

Evaluation of acetyl cholinesterase activity

Acetyl cholinesterase (AChE) activity was determined in cell extracts of experimental groups using acetylcholine iodide as a substrate according to the method of Ellman [12]. In this method AChE in samples hydrolyzes acetyl thiocholine iodide into thiocholine and butyric acid. The thiocholine reacts with 5, 5′-dithiobis-2-

nitrobenzoic acid to form 5-thio-2-nitrobenzoic acid. The yellow colour developed is measured spectrophotometrically at 412 nm (PIOWAY, Focus Technology Co., Ltd.).

Evaluation of oxidative stress markers

The concentration of thiobarbituric acid reactive substances (TBARS) were estimated in cell extracts of experimental groups by the method of Niehaus and Samuelsson [13] using 1,1′,3,3′-tetramethoxypropane as the standard. Lipid hydroperoxides (LOOH) in cell extracts of experimental groups were assayed as described by Jiang [14].

Measurement of mitochondrial function markers

As mitochondrial function markers, we chose mitochondrial membrane potential (MMP) and cytochrome-c oxidase activity. MMP was evaluated using the JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical Company, Ann Arbor, Michigan, USA). Cytochrome-c oxidase activity in cell homogenates was assessed using a cytochrome-c oxidase assay kit (Sigma Aldrich, China) adapted to a Cobas Mira Autoanalyser [15]. One unit was defined as the oxidation of 1.0 µmol of ferrocytochrome c per minute at pH 7.0 and 37 °C.

Evaluation of antioxidant levels

The cells were washed with ice-cold phosphate buffered saline (PBS) and were lysed with RIPA buffer (Cell Signaling Technology). The lysates were centrifuged, and the protein content of the supernatant was determined by the Bradford method [16]. About 10–20 µg of protein was loaded and separated by 10 % SDS–PAGE and blotted onto a polyvinylidene fluoride membrane. Then, the membranes were blocked with 5 % non-fat dry milk powder solution for 1 h at room temperature before an overnight incubation with primary antibodies (catalase (CAT), glutathione peroxidase (GPx) and superoxide dismutase (SOD) (purchased from BD Biosciences) at 4 ^{o}C. After rinsing the membranes, they were incubated with secondary antibody for 1 h at room temperature and finally developed through Alpha InfoTech Fluor Chem. HD2 Imaging System (Alpha Innotech). All antibodies, with the exception of α-tubulin, were used at a 1:250 dilution. The antibodies of α-tubulin were used at a 1:1000 dilution.

Statistical analysis

The data are expressed as mean ± standard deviation (SD) for six animals in each group. Differences between groups were assessed by a one-way analysis of variance (ANOVA) using SPSS software package for Windows (Version 11.5; SPSS Inc., Chicago, IL, USA). Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test.

RESULTS

IGF-II prevents MPP- induced ROS production and cell death

The cells exposed to 10 µM of MPP (G1) showed a significant increase in percentage of ROS production and cell death. However, cells exposed to MPP and treated with 100 ng/mL of IGF-II (G2) had significantly reduced ROS production (58.33 %, $p < 0.001$) and cell death (76 %, $p < 0.04$) when compared to G1 (Figure 1). Protective effect was observed in cells exposed to MPP and treated with IGF in the presence of 20 ng/µL IGF-I analogue (G3) or 5 ng/µL anti-IGF-IIR (G4). However, an increase in ROS (50 %) and cell death (77.14 %) was observed in the cells exposed to MPP in the presence of IGF-II with both inhibitors of IGF receptors (G5) Vs G2.

Activities of AChE and oxidative stress markers

The activity of AChE in cortical neuronal cells exposed to MPP was found significantly (61.11 %, $p < 0.05$) decreased when compared to control (Figure 2a). However, this MPP mediated-toxicity was controlled by the administration of IGF-II (50 %, G2 Vs G1). Consistently, IGF-II treatment had significantly decreased the levels of MPP-induced oxidative markers (TBARS-40 %; LOOH- 39.28 %: G2 Vs G1) (Figure 2b). The inhibition study also showed decrease in the oxidative marker suggesting the contributions of IGF receptors in IGF-II treatment in neural cells against MPP exposure (Figure IIb).

IGF-II improved the activity of mitochondrial markers

The cells exposed to MPP showed a significant decrease in MMP and cytochrome-C oxidase activity (Figure 3). However, IGF-II treatment restored these activities to near normal. No

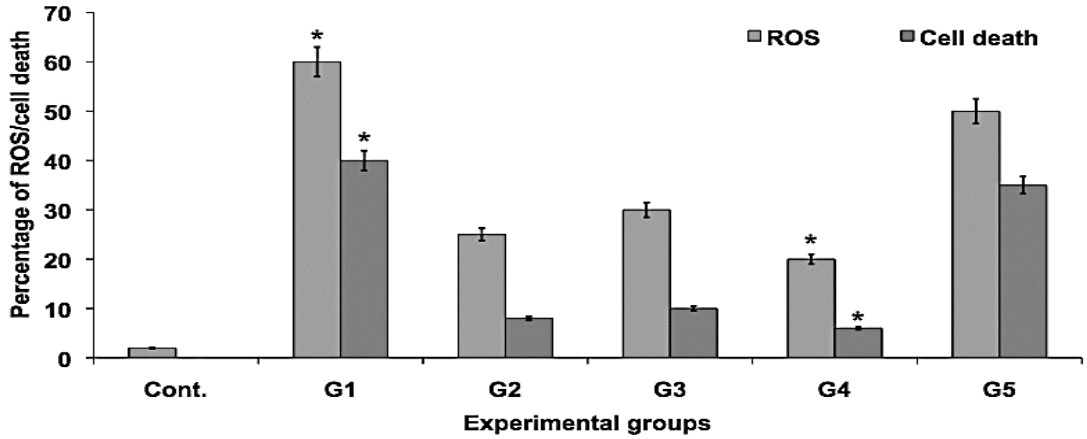

Figure 1: Graphical representation showing percentage of ROS production and cell death in adult cortical neuronal cells in the experimental groups (G) as mentioned in **Table I.** *- Indicate the significant (p < 0.05) difference from IGF-II treated group (G2).

Table 1: Cell culture treatment and experimental grouping

Cell treatment	Experimental Groups (G)					
	Cont.	G1	G2	G3	G4	G5
1-methyl-4-phenyl pyridinium (MPP; 10 µM)	-	+	+	+	+	+
IGF-II (100 ng/mL)	-	-	+	+	+	+
IGF-I analogue (20 ng/µL)	-	-	-	+	-	+
Anti-IGF-IIR (5 ng/µL)	-	-	-	-	+	+

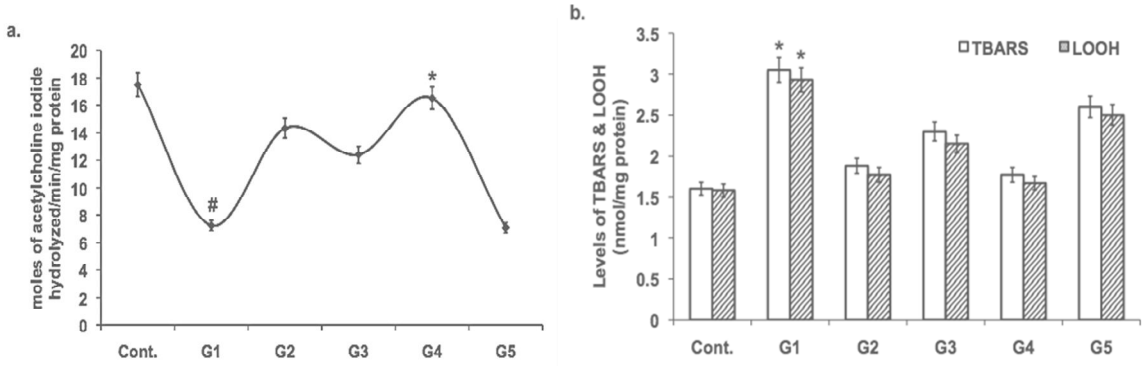

Figure 2: (a) Activity level of acetylcholine esterase measured in the experimental groups (G) as mentioned Table I. # Indicate the significant (p < 0.001) difference from IGF-II treated group (G2); *indicate the significant (p < 0.05) difference from IGF-I analogue (IGF-IR inhibitor) treated group (G3). b. Represent the level of oxidative stress or lipid peroxidation markers TBARS and LOOH in the experimental groups. * -Indicate the significant (p < 0.001) difference from IGF-II treated group (G2)

changes were detected upon incubation in MPP, IGF-II and either inhibitor of IGF receptors, whereas co-incubation with both inhibitors restored the MMP/ cytochrome-c oxidase activities to that of the MPP-treated cells (Fig 3).

IGF-II improved the antioxidant level

MPP-exposed cells showed significantly (p < 0.05) decreased protein levels of CAT (60.86 %), SOD (61.11 %) and Gpx (70 %), which were restored by treatment with IGF-II 55, 56.25 and 70 %, respectively (Figure 4a-c). While as IGF inhibition also showed the protective effect individually, there was, however, little or no protective effect when administered with both inhibitors (IGF-1 analogue and anti-IGF-IIR) during MPP exposure (Figure 4a-c).

DISCUSSION

IGF-II remains the most abundant insulin-like peptide in the adult brain, primarily expressed in the choroid plexus, the leptomeninges and the

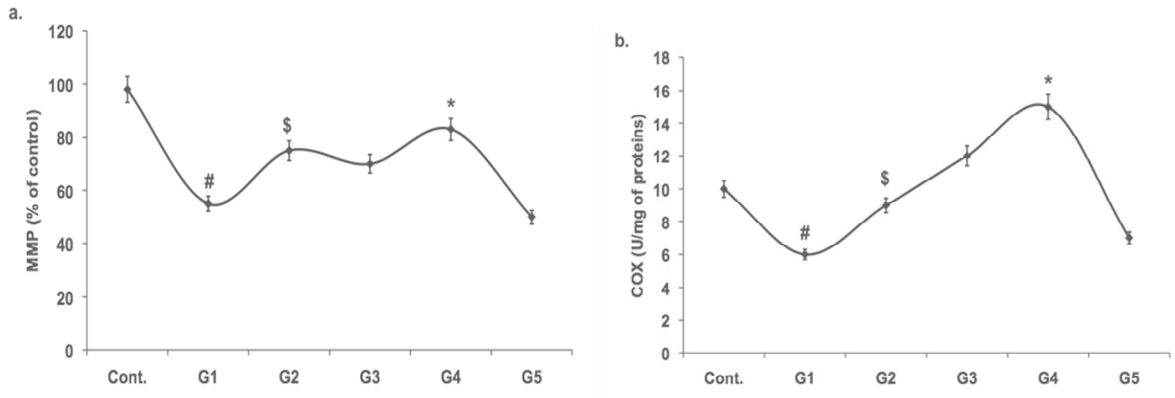

Figure 3: Graphical representation showing the activity of mitochondrial functional markers MMP (a) and cytochrome-c oxidase (b) in the experimental groups. # Indicate significant (p <0.001) difference from control group; $ indicate significant (p < 0.001) difference from MPP-exposed group (G1); *indicate significant (p < 0.05) difference from IGF-I analogue (IGF-IR inhibitor) treated group (G3)

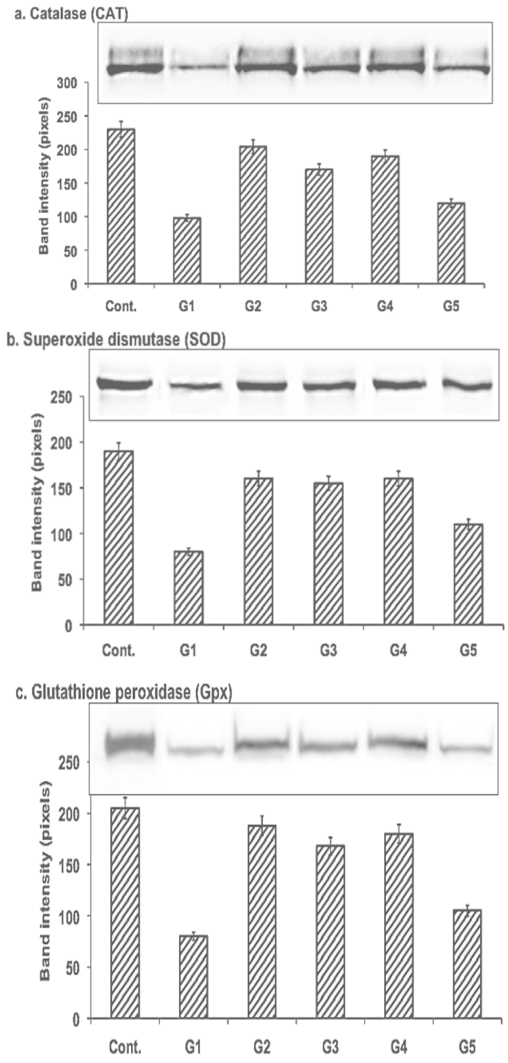

Figure 4: Western blot data for antioxidant enzymes: (a) catalase, (b) superoxide dismutase and (c) glutathione peroxidase in the experimental groups. Band intensities were quantified, calibrated/normalized with the bands of α-tubulin and represented in the bar graph

hypothalamus [10]. IGF-II is capable of activating the IGF receptors (IGF-IR and IGF-IIR), in which,IGF-IIR is the most abundant insulin-like receptor in the adult brain. The physiological actions of IGF-II in adults are not well defined, although some studies have demonstrated an association of these peptides with cognitive processes, such as memory [17], and neuro-protection against neurodegenerative disorders. The present study was focused towards the protective effects of IGF-II and the supporting factors involved in the IGF-II mediated neuro-protection. The cultures of cortical neurons from adult rats were treated with high concentration of MPP for the quick induction of oxidative stress or neurotoxicity. The low concentration of IGF-II was chosen based on the results of previous experiments that demonstrated the capacity of IGF-II to induce neuroprotection by reducing oxidative stress in the cortex and the hippocampus of aged rats [18].

Excessive amounts of ROS and mitochondrial dysfunction are among the major mechanisms that trigger neurodegeneration [19]. In the present study, the percentage of ROS production and the cell death was significantly increased in MPP-exposed cells, and it was found that IGF-IIR plays major role than that of IGF-IR. Similar effects of IGF-II on these oxidative markers have also been reported in the aged brain (4). To reveal the supportive factors for IGF-II mediated protection (7), we incorporated the study on IGF receptors specific inhibitors (IGF-1 analogue and anti-IGF-IIR). The results showed the protective effect of IGF-II against MPP in individual inhibitor treatment but no protective effect was found in simultaneous treatment of both inhibitors. These results clearly predict the involvement of IGF receptors in IGF-II mediated neuroprotection, which is consistent with the previous reports [20].

Numerous studies have suggested that the free radical production could at least associate with the decreased activity of brain AChE [21]. The brain AChE activity is an important regulator of the behavioral processes; in the present study, the decreased level of AChE in neuronal cells might be one of the indicators for MPP induced toxicity, whereas IGF-II treatment restored the activity of AChE. In addition, we have evaluated the effect of IGF-II on MPP-induced oxidative stress markers (TBARS and LOOH), which are related to damage to lipid membrane. The elevated level of TBARS and LOOH was decreased to near control levels. To assess mitochondrial function, oxidative phosphorylation was evaluated by measuring the MMP [22] and mitochondrial cytochrome-c oxidase activity, which is the rate-limiting step of the mitochondrial respiratory chain [23]. JC-1 fluorescence was measured which reflects the average MMP of all mitochondria inside the cells. Both MMP and cytochrome-c oxidase activity was restored upon IGF-II treatment against MPP exposure. Mitochondrial membrane potential (MMP) and cytochrome-c oxidase activity were measured as the markers of respiratory chain to determine the mitochondrial function. Our inhibitor study on MMP and cytochrome-c oxidase further supported the involvement of IGF receptors in the protective effect of IGF-II. In addition, IGF-II protected the MPP-induced decrease in antioxidant protein levels of CAT, SOD and Gpx in neuronal cells, which is supported by the involvement of IGF receptors. Our study revealed that IGF-II functions under the influence of IGF receptors and in particular view our results suggest that IGF-IIR involvement is more efficient than that of IGF-IR.

CONCLUSION

IGF-II exhibits a neuroprotective effect on MPP-exposed cortical neuronal cells. The results reveal that the protective effect of IGF is supported by the involvement of IGF-II activated IGF receptors. Thus, IGF-II may be a good therapeutic target for various neurodegenerative disorders such as Parkinson's disease. However, further elucidation of the exact molecular mechanism is required.

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