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Neuroprotective effects of LMW and HMW FGF2 against amyloid beta toxicity in primary cultured hippocampal neurons

Yong Cheng¹, Zhaojin Li¹, Elissavet Kardami², and Y. Peng Loh^{1,*}

¹Section on Cellular Neurobiology, Program on Developmental Neuroscience, Eunice Kennedy Shriver National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Md. 20892, U.S.A

²Institute of Cardiovascular Sciences, St. Boniface Hospital Albrechtsen Research Centre, University of Manitoba, Winnipeg, Manitoba, Canada

Abstract

Basic Fibroblast growth factor (FGF2) is important in development and maintenance of central nervous system function. Studies have demonstrated that low molecular weight (LMW) FGF2 is a neuroprotective factor against various insults in vivo and in vitro. In the present study we investigated the neuroprotective effects of high molecular weight (HMW) and LMW FGF2 against amyloid beta-induced neurotoxicity. The results showed that both LMW and HMW FGF2 attenuated the amyloid beta toxicity in the primary cultured hippocampal neurons as measured by WST and LDH release assay. Moreover, the analysis suggested that HMW FGF2 had stronger neuroprotective effect than LMW FGF2. We then demonstrated that LMW and HMW FGF2 activated the ERK and AKT signaling pathways in a similar way. Furthermore, using the ERK inhibitor and AKT inhibitor, we found that the AKT signaling but not ERK signaling pathway was required for the neuroprotective effects of FGF2. Taken together, these results showed the neuroprotective effects of different forms of FGF2 in an AD model and the mechanism underlying the neuroprotection.

Keywords

Hippocampal neuron; HMW FGF2; LMW FGF2; Amyloid beta; AKT

1. Introduction

Alzheimer's disease (AD) is the most common dementia in the elderly population and it was the sixth leading cause of deaths in the United States in 2010 [1]. Although the etiology of AD is still poorly understood, the amyloid hypothesis is the most prevalent one. The amyloid hypothesis postulated that the extracellular amyloid beta accumulation in the brain

*Send Correspondence to: Dr. Y. Peng Loh, Bldg. 49, Rm. 6A-10, National Institutes of Health, 49 Convent Drive, Bethesda, MD 20892, USA. loh.p@mail.nih.gov, Tel.: (301) 496-3239, Fax: (301) 496-9938.

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is the primary influence driving AD pathogenesis [2]. Indeed, numerous studies demonstrated that amyloid beta caused neurotoxicity in vitro and in vivo [3–5]. Thus amyloid beta toxicity is the primary target for potential treatment of AD.

Fibroblast growth factor 2 (FGF2), also known as basic FGF, has pleiotrophic effects in different tissues and organs [6]. FGF-2 has several isoforms resulting from alternative initiations of mRNA translation in rodents, a 17/18 KDa isoform mainly present in cytoplasm and two higher molecular weight isoforms (21 and 23 KDa) mainly present in nucleus [7]. In addition, both low molecular weight and high molecular weight FGF2 were found in the extracellular space [8,9]. It is well known that FGF2 is important both in development and maintenance of nervous system [10]. Furthermore, studies have suggested that FGF2 plays a role in neuropsychiatric diseases such as depression and AD. FGF2 has been linked to major depression, since it is down-regulated in serum of patients with major depressive disorder compared to normal controls [11]. Recent studies have demonstrated exogenous FGF2 rescued depression-like behavior in mice [12] and anxiety-like behavior in rats [13] by promoting hippocampal neurogenesis. In AD, a study showed that delivery of FGF2 gene to hippocampus restored the hippocampal functions in mouse models of AD [14]. Another study demonstrated that subcutaneous injection of FGF2 reduced BACE1 expression and amyloid pathology in APP23 transgenic mice [15]. The above results suggested that FGF2 could have potential therapeutic applications in various neuropsychiatric diseases. However, these studies have focused on the role of low molecular weight FGF2 in nervous system.

In the present study, we explored the neuroprotective effects of different forms of FGF2 against amyloid beta toxicity in primary cultured hippocampal neurons and the mechanism underlying it.

2. Materials and Methods

2.1. Primary hippocampal neuron culture

Hippocampal neuronal cultures from embryonic E18 rats were prepared as described previously [16]. Briefly, the hippocampus was dissected, digested and dissociated into single cells. Then the cells were plated on poly-L-lysine (Sigma, St. Louis, MO, USA) coated plates at a density of 1×10^6 cells/ml. After over-night plating, the medium was replaced by neurobasal medium with 2% B27 (Invitrogen, Carlsbad, CA, USA). The hippocampal neurons were treated under various conditions after at least five days in culture.

2.2. Reagents

Recombinant rat HMW FGF2 (23 kDa) and LMW FGF2 (18 kDa) were produced in *Escherichia coli* bacteria and purified by affinity chromatography as described previously [17,18]. A β _{1–42} (Peptide 2.0 Inc, Chantilly, VA, USA) was dissolved in DMSO and stocked at a concentration of 5 mM. SU5402 was purchased from Sigma, U0126 and LY294002 were from Cell Signaling (Danvers, MA, USA)

2.3. WST-1 assay

Water soluble tetrazolium salts-1 (WST-1) Cell Proliferation Reagent (Clonotech, Mountain View, CA, USA) assay was used to determine the viability of the primary cultured hippocampal neurons after various treatments as described previously [19]. Briefly, WST-1 reagent was added to the neurons, and cells were incubated with it for 1–2 h at 37°C in a CO₂ incubator, then the absorbance at wavelength of 450 nm was measured using a microplate reader.

2.4. LDH release assay

Lactate Dehydrogenase (LDH) release assay was used to measure the cytotoxicity of cells after various treatments. This was achieved with a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit following the manufacturer's protocol (Promega, Madison, WI).

2.5. Western blot

Western blot was used to determine the phosphorylation levels of ERK and AKT as described previously [16]. Briefly, soluble protein in hippocampal neurons in culture were extracted from lysates and twenty µg of protein from the supernatants were analyzed by standard Western blotting procedures using nitrocellulose membrane. The Odyssey infrared imaging system (LI-COR Inc, Lincoln, NE, USA) was used to detect the protein bands. Monoclonal mouse anti-p-AKT S473 antibody (1:3000), polyclonal rabbit anti-t-AKT antibody (1:5000) were from Cell Signaling. Monoclonal mouse anti-p-ERK antibody (1:1000) and polyclonal rabbit anti-t-ERK antibody (1:5000) were from Santa Cruz (Dallas, Texas, USA).

Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey post-hoc multiple comparisons tests, or two-way ANOVA where noted. Significance was set at $p < 0.05$.

3. Results

3.1. FGF2 attenuated A β _{1–42} induced neurotoxicity

To investigate the neuroprotective effects of FGF2, primary cultured hippocampal neurons were treated with 20 µM A β _{1–42} in the presence or absence of different concentrations of FGF2 for 24h, then WST assay and LDH release assay were used to measure the cell viability and cytotoxicity. As shown in Fig 1A and 1B, A β _{1–42} significantly reduced the cell viability and increased cytotoxicity in the primary cultured hippocampal neurons, which is consistent with previous studies. The results also showed that both LMW and HMW FGF2 at 10 µg/ml, 50 µg/ml and 200 µg/ml significantly inhibited the A β _{1–42} induced neurotoxicity in the neurons, the neuroprotective effect of FGF2 reached the plateau at 50 µg/ml (One way ANOVA analysis). Furthermore, we used two way ANOVA analysis to compare the effects of LMW and HMW FGF2. As compared to LMW FGF2, HWM FGF2 had more potent neuroprotective effect against A β toxicity in the primary cultured hippocampal neurons (Fig 1C and 1D).

3.2. FGF receptor mediated the neuroprotection of FGF2

To find out whether FGF receptor mediated the neuroprotective effects of LMW and HMW FGF2, we treated the hippocampal neurons with the FGF receptor 1 inhibitor, SU5402. As shown in Fig 2, 100 nM SU5402 blocked the neuroprotective effects of both LMW and HMW FGF2 against A β toxicity, demonstrating that both forms of FGF2 can act on the receptor to play the neuroprotective role in the primary cultured hippocampal neurons.

3.3. AKT signaling pathway was required for the neuroprotection of FGF2

We next investigated which signaling pathways are involved in the neuroprotection of FGF2 against A β ₁₋₄₂. We tested ERK and AKT signaling pathways as both are major pathways for cell survival and neuroprotection [20–22]. The primary cultured hippocampal neurons were treated with LMW FGF2, HMW FGF2 or vehicle for 30 min, then western blot was used to measure the activation of ERK and AKT. As shown in Fig 3A, both forms of FGF2 activated ERK in a similar manner in the neurons. Similar results were obtained in the activation of AKT by FGF2 (Fig 3B).

To find out whether the ERK and AKT signaling pathways are mediating the neuroprotection of FGF2, we used the ERK inhibitor-U0126 and AKT inhibitor- Ly294002. As shown in Fig 4A, 1 μ M U0126 did not have any effect on the neuroprotection of FGF2. In contrast, 10 μ M Ly294002 completely abrogated the neuroprotective effect of both forms of FGF2 against A β toxicity (Fig 4B). These results suggested that AKT, but not ERK signaling pathway mediated the neuroprotection of FGF2 in the primary cultured hippocampal neurons.

4. Discussion

FGF2 belongs to a large family of heparin-binding proteins, and it can be synthesized to LMW FGF2 (18 kDa) or HMW FGF2 (>20 kDa) [9]. All forms of FGF2 are from a single mRNA, translation from AUG or CUG start codons produces LMW-FGF-2 (17/18 kDa) or HMW-FGF-2 (21–23 kDa in rat or mouse; 21–34 kDa in human) [23]. The biological functions of LMW FGF2 have been extensively studied, HMW FGF2 is not well studied although gaining more attention gradually [24]. Some studies highlighted the distinct roles of different forms of FGF2. It has been reported that after myocardial infarction in rats, LMW FGF2 promoted sustained cardioprotection and angiogenesis, while HMW FGF2 promoted myocardial hypertrophy and reduced contractile function [23]. In an animal model of Parkinson's disease, co-transplanted dopamine grafts with Schwann cells engineered to overexpress HMW FGF2 showed enhanced restoration compared to LMW FGF2 [25]. In another study by the same group they showed that HWM FGF2 promoted more neurotrophic activity on rat embryonic mesencephalic dopaminergic neurons *in vitro* [26]. In our study, we demonstrated that recombinant FGF2 protected against amyloid beta toxicity in primary cultured hippocampal neurons, consistent with the previous report that FGF2 attenuated the neurotoxicity caused by amyloid beta in embryonic rat septal neurons [27]. Our analysis also indicated that HMW FGF2 had enhanced neurotrophic activity compared to LMW FGF2, suggesting the differential effects of HWM and LMW FGF2 in an AD model. In addition to FGF2, other neurotrophic factors such as brain-derive neurotrophic factor (BDNF) and nerve

growth factor (NGF) had also been demonstrated to be neuroprotective in AD models [28,29]. Additionally, BDNF levels were found to be decreased in the patients with AD [30], and the maturation process of NGF was compromised in AD [31], suggesting the potential role of neurotrophic factors in the progression of AD. Therefore, it would be interesting to study whether the expression or activity of FGF2 is abnormal in patients with AD.

Both types of FGF2 isoforms are found in the extracellular space [8,9], and therefore both isoforms are expected to exert effects by binding and activating plasma membrane FGF receptor. In this study, using SU5402, a FGF receptor 1 inhibitor we have demonstrated that the neuroprotective effects of both forms of FGF2 were abolished by the inhibitor, indicating that FGF2 activated the receptor to exert neuroprotection. These results are supported by the studies showing both HMW and LMW isoforms can activate signal transduction pathways via plasma membrane FGF receptor [32,33].

It is known that FGF2 can activate ERK and AKT in the primary cultured hippocampal neurons [34]. In addition, both ERK and AKT signaling pathways are well known for the major roles in mediating the neuroprotective actions of neurotrophic factors [20–22]. In the present study we confirmed that FGF2 activated the ERK and AKT signaling pathways in the primary cultured hippocampal neurons. Our results further showed that HMW and LMW FGF2 activated ERK and AKT in a similar way in the neurons. Furthermore, pretreatment with the AKT inhibitor (Ly294002), reduced the neuroprotection of FGF2 against amyloid beta. In contrast, pretreatment with the ERK inhibitor, U0126, did not have effect on the neuroprotection induced by FGF2. Thus our data revealed that AKT but not ERK signaling pathway was involved in the FGF2 mediated neuroprotection in an AD model, this is consistent with the previous study showing that AKT is required for FGF-2-stimulated survival in primary cultured hippocampal neurons [34]. Since our data did not show significant difference between HMW and LMW FGF2 mediated ERK and AKT activation, the stronger neuroprotective effect induced by HMW FGF2 may be mediated by the other signals. In fact, in the perfused isolated hearts, administration of HMW FGF2 after ischemia and during reperfusion, was as protective as LMW FGF2, but elicited stronger activation of the p70S6 kinase and the PKC-zeta kinase [35]. In addition, HMW FGF2 can potentially engage additional plasma membrane receptors, such as neuropilin-1, via its N-terminal extension domain in different cell types [36].

In conclusion, this study demonstrated the neuroprotective effects of different forms of FGF2 in an AD model and the mechanism underlying it. Further investigations into different forms of FGF2 as potential therapeutic targets of AD are warranted.

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Highlights

- LMW FGF2 and HMW FGF2 protected against A β ₁₋₄₂- induced neurotoxicity in the neurons.
- HMW FGF2 had stronger neuroprotective effect
- The AKT signaling pathway mediates the neuroprotective effect of FGF2
- Differential effects of different forms of FGF2 should be considered in future studies.

Fig. 1.

Neuroprotective effects of LMW and HMW FGF2 on Aβ₁₋₄₂ toxicity in the primary cultured hippocampal neurons. (A) WST assay showing that the reduced cell viability induced by Aβ₁₋₄₂ was inhibited by LMW or HMW FGF2 in a dose dependent manner in the primary cultured hippocampal neurons. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=26.58$, $p<0.05$. * compared to the control group, # compared to Aβ₁₋₄₂ group. (B) LDH release assay showing that LMW or HMW FGF2 reduced the cytotoxicity induced by Aβ₁₋₄₂ in the neurons. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=10.4$, $n=5$, $p<0.05$. * compared to the control group, # compared to Aβ₁₋₄₂ group. (C, D) Two way ANOVA analysis showing that HMW FGF2 had stronger neuroprotection than LMW FGF2 in the primary cultured hippocampal neurons. For WST assay: $F=30.90$, $n=5$, $p<0.05$; for LDH release assay: $F=10.4$, $n=5$, $p<0.05$. Three independent experiments were done, data shown represent one experiment.

Fig. 2.

FGF receptor is required for the neuroprotection of FGF2. LDH release assay showing that the neuroprotective effects of HMW and LMWFGF2 against Aβ₁₋₄₂ were abolished by the FGF receptor 1 inhibitor (SU5402) in the neurons, indicating that FGF receptor activation was required for the neuroprotective effects of FGF2. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=20.94$, $n=5$, $p<0.05$. Two independent experiments were done, data shown represent one experiment.

Fig. 3.

LMW and HMW FGF2 activate ERK and AKT in the neurons. (A) Representative western blot bands of phosphorylated ERK after 30 min treatment with FGF2 in the hippocampal neurons. Total ERK served as internal control. (B) Quantification of p-ERK normalized to t-ERK. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=7.587$, $n=4$, $p<0.05$. * compared to the control group. (C) Representative western blot bands of phosphorylated AKT after 30 min treatment with FGF2 in the hippocampal neurons. Total AKT served as internal control. (D) Quantification of p-Akt normalized to t-ERK. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=11.89$, $n=4$, $p<0.05$. Data were pooled from two independent experiments.

Fig. 4.

Activation of AKT but not ERK is required for the neuroprotection of FGF2. (A) LDH release assay showing that the rescue effect of FGF2 after Aβ₁₋₄₂ induced damage in the neurons was not blocked by ERK inhibitor (U0126), suggesting that ERK activation was not involved in the neuroprotection of FGF2. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=19.83$, $n=4$, $p<0.05$ (B) LDH release assay showing that the rescue effect of FGF2 after Aβ₁₋₄₂ induced damage in the neurons was blocked by AKT inhibitor (Ly294002), suggesting that AKT activation was involved in the neuroprotection of FGF2. One way ANOVA followed by Tukey post-hoc multiple comparisons tests, $F=21.71$, $n=5$, $p<0.05$. Two independent experiments were done, data shown represent one experiment.