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Possible involvement of induction of brain-derived neurotrophic factor in the neuroprotective effect of a 5-phenylpyrimidine derivative

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Abstract

When primary cortical neurons prepared from the brains of rat embryos (E18) were cultured in the absence of serum, most of the neurons died after 3 days *in vitro*. We used this model to discover compounds which support neuronal survival, and found that a new 5-phenylpyrimidine derivative named FU248 (2-amino-5-(2,4-dichlorophenyl) pyrimidine) inhibited the neuronal cell death in a dose-dependent manner up to 1 μg/mL. Semiquantitative RT–PCR analysis revealed that an exposure of the primary cortical neurons to 1 μg/mL of FU248 transiently and significantly enhanced the expression of genes including brain-derived neurotrophic factor (BDNF), nerve growth factor (NGF), and neurotrophin-3 (NT-3). The enhancement of the gene expression was maximal 6 hr after the addition of FU248, and the expression returned to the basal level after 24 hr. Expression of neurotrophin-4 was not detectable throughout the experimental period. The amount of the transcript for BDNF was approximately nine times and sixteen times more abundant than those for NT-3 and NGF, respectively ($t = 6$ hr). Moreover, an anti-BDNF antibody suppressed the effect of FU248, whereas the control antibody did not show any effects on the neuronal survival. These findings strongly suggest that FU248 exerts its neuroprotective effect, at least in part, through induction of BDNF.

Keywords: 5-Phenylpyrimidine; Brain-derived neurotrophic factor; Primary cortical neuron; Neuronal survival; RT–PCR

1. Introduction

Rat primary cortical neurons are well established and useful model to study neuronal cell death caused by cerebral ischemia. The neurons cannot survive without trophic support, and cell death can be induced by several methods such as serum deprivation. We have employed this model for random screening of our library of low molecular weight compounds to develop drugs which inhibit neuronal cell death caused by cerebral ischemia, and discovered several compounds which supported survival of primary cortical neurons in the absence of serum. One of such compounds was a new 5-phenylpyrimidine derivative named FU248 (2-amino-5-(2,4-dichlorophenyl) pyrimidine). We had synthesized 5-phenylpyrimidine derivatives originally aiming at new nonsteroidal anti-inflammatory drugs. In fact, several 5-phenylpyrimidine derivatives significantly inhibited carrageenin-induced rat paw edema. FU248 also showed a slight anti-inflammatory activity, but the activity was not significant [1]. Interestingly, however, we found that FU248 had a neuroprotective activity in the current study. In this report, we will describe that FU248 exerts its neuroprotective effect, at least in part, through induction of BDNF.

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Abbreviations: BDNF, brain-derived neurotrophic factor; DMEM, Dulbecco's modified MEM; FBS, fetal bovine serum; FDA, fluorescein diacetate; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein-2; NGF, nerve growth factor; NT-3/4, neurotrophin-3/4; PBS, phosphate-buffered saline.

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2. Materials

DMEM and Ham's F12 medium were purchased from Gibco BRL. DF medium is a 7:3 mixture of DMEM and F12 medium. FBS was obtained from CSL, Ltd. All other supplements required for cell culture were purchased from Sigma. FDA was purchased from Calbiochem.

An anti-BDNF antibody (lot no. 076H051) was purchased from Pepro Tech EC, and mouse monoclonal antibodies against MAP-2 and GFAP were purchased from Boehringer Mannheim Biochemica.

FU248 was synthesized and purified by Hibino and coworkers as described previously [1,2].

3. Methods

3.1. Cell culture

Primary cortical neurons were prepared from the brains of E18 Wistar rat embryos using methods described by others [3,4] with a slight modification. In brief, cortices of the brains were dissected, and the tissue fragments were treated with 0.25% trypsin and 0.1% DNase I (Sigma) for 15 min at 37° and passed through Nylon mesh (150 μm) to prepare single cells. Dissociated cells were resuspended in DF medium containing 10% FBS, and plated at a density of 5×10^5 cm^{-2} in a 24-well plate (Corning Costar) previously coated with polyethylenimine (Sigma). After culturing for 24 hr at 37° in a humidified CO_2 (5%) incubator, the medium was changed to serum-free DF medium supplemented with 5 ng/mL of sodium selenite, 20 nM of progesterone, and 5 μg/mL each of insulin and transferrin (TIP/DF medium). After 3 days of culture *in vitro*, the cells were incubated with 15 μg/mL of FDA (Sigma) for 10 min at room temperature. The intensity of fluorescence (excitation 485 nm/emission 530 nm) was measured with CytoFluor2300 (Millipore) for quantification of living cells [5]. Results were statistically analyzed by ANOVA with *post hoc* Fisher's PLSD.

3.2. Immunohistochemistry

The cultured rat cortical neurons were fixed with 4% paraformaldehyde for 10 min at room temperature. Mouse monoclonal antibodies against MAP-2 and GFAP were used as the first antibody at the final concentration of 10 μg/mL. Ventana NX System (Ventana Japan) was used for all the immunohistochemical analyses.

3.3. Semiquantitative RT–PCR

Total RNA was extracted from the cultured cortical neurons using TRIzol (Gibco BRL), further purified with RNeasy (QIAGEN), and treated with RNase-free DNase (Gibco BRL) prior to reverse transcription. cDNAs for NGF, BDNF, NT-3, and NT-4 were amplified with primer sets of the following sequences:

NGF: 5′CCACTGGACTAAACTTCAGCATTCCC (sense) and 5′AACGCCTTGACAAAGGTGTGAGTCGT (antisense);
BDNF: 5′ACCAGGTGAGAAGAGTGATGACCATC (sense) and 5′AGTGTCTATCCTTATGAACCGCCAGC (antisense);
NT-3: 5′GTGAACAAGGTGATGTCCATCTTGTTT (sense) and 5′TTTTTGACCGGCCTGGCTTCCTTACAT (antisense); and
NT-4: 5′CCCAGTGTGCCAATTGAGTCCCAA (sense) and 5′TTCAAAGAAGTACTGGCGGAGGGGA (antisense).

As a control, a transcript for S26 ribosomal protein was amplified [6] with a primer set of the following sequences: 5′AAAAGAAGGAACAATGGTCGTGCC (sense) and 5′TCAGCTCCTTACATGGGCTTTGG (antisense).

Each reaction was performed in the presence of 0.6 MBq of [α-^{32}P]dCTP (222 TBq/mmol; Amersham) under the following conditions: 90°, 30 s; 60°, 30 s; 72°, 1 min, for 18 cycles. The lengths of PCR products for NGF, BDNF, NT-3, NT-4, and S26 are 528, 728, 620, 365, and 347 bp, respectively. The PCR products were then separated through a 10% polyacrylamide gel. After drying the gel on a filter paper, the radioactivity of each band was quantified with BAS2000II Bio Image Analyzer (Fuji

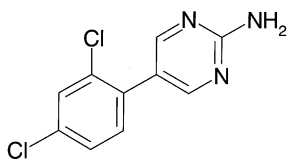

Fig. 1. Molecular structure of FU248.

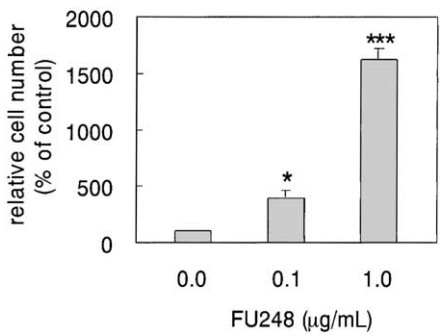

Fig. 2. Effects of FU248 on survival of rat primary cortical neurons. Primary cortical neurons prepared from the brains of E18 rat embryos were cultured for 3 days *in vitro* in TIP/DF medium in the presence of various concentrations of FU248 or 0.1% DMSO as a vehicle control. Surviving neurons were quantified by FDA method. The relative cell numbers were shown as percentages of the control, where no trophic support was added. Each data bar represents a mean ± SEM ($N = 4$). Significance was determined using the value of the control ($^{*}P < 0.05$, $^{***}P < 0.001$).

Film) and normalized to the number of deoxycytidine residues in the PCR products. The expression level of each gene was further normalized to that of S26.

4. Results

4.1. Effects of FU248 on survival of rat primary cortical neurons

Primary cortical neurons were prepared from the brains of rat embryos (E18) by an established method and cultured for 3 days *in vitro* in the absence of serum. The neurons could not survive under these conditions, and only less than 10% of the neurons survived after 3 days of culture *in vitro* by our hands (data not shown). We employed this model to look for low molecular weight compounds which support neuronal survival, and discovered a new 5-phenylpyrimidine derivative named FU248 (Fig. 1). When added throughout the incubation period, FU248 supported survival of rat primary cortical neurons *in vitro* in a dose-dependent manner up to 1 μg/mL (Fig. 2). Immunohistochemical staining revealed that 91.8 ± 0.6% of the surviving cells were actually MAP-2 positive neurons (Fig. 3A), though some of the cells were consistently stained with an anti-GFAP antibody (Fig. 3B).

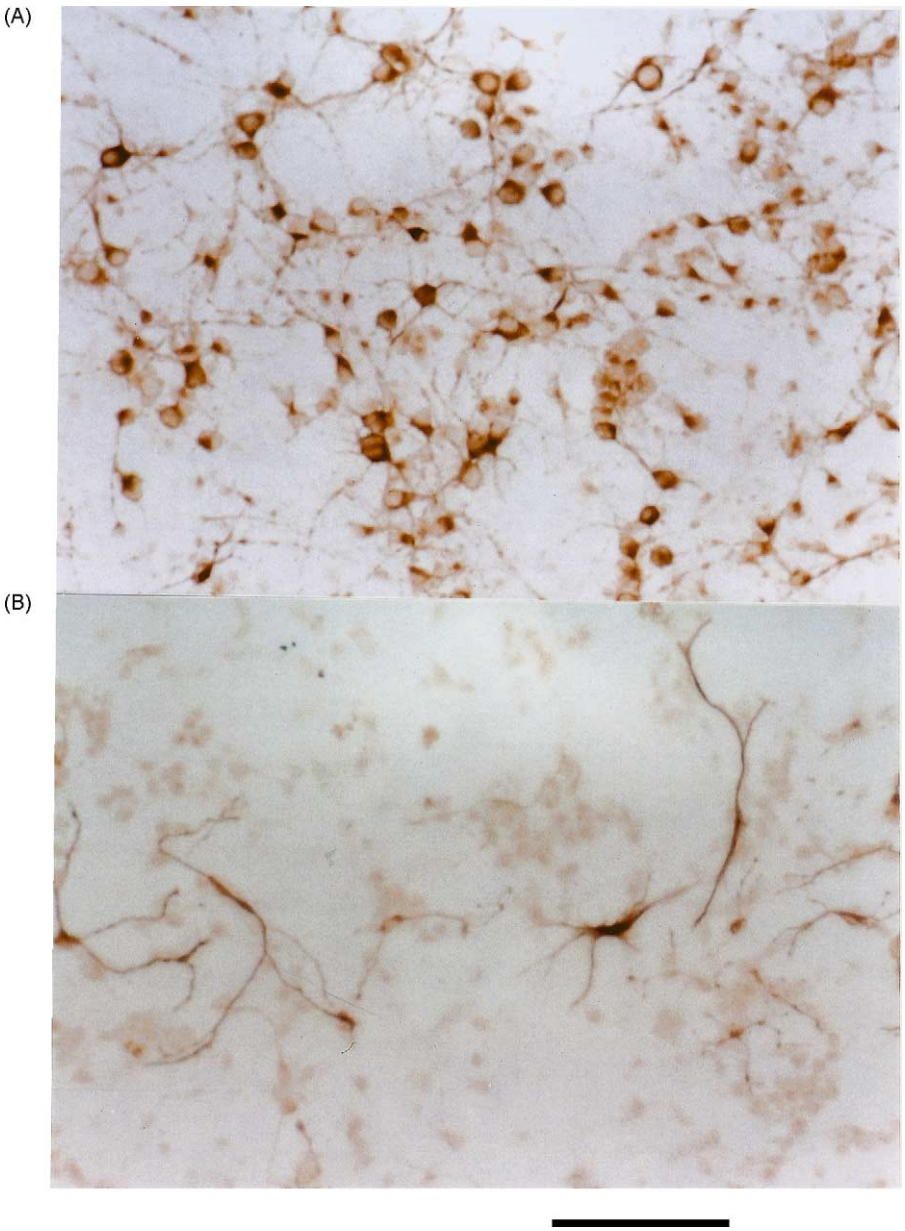

Fig. 3. Immunohistochemical analysis of surviving neurons. Primary cortical neurons were cultured on glass slips in TIP/DF medium for 3 days *in vitro* in the presence of 1 μg/mL of FU248. Surviving neurons were stained with either an anti-MAP-2 (A) or an anti-GFAP (B) antibody and observed under a light microscope. Bar shows 100 μm.

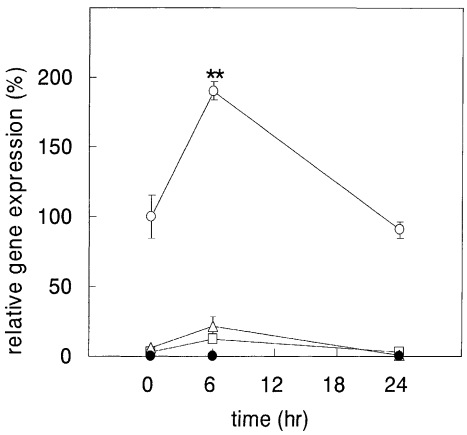

Fig. 4. Gene expression profile of neurotrophins induced by FU248. Primary cortical neurons were exposed to 1 μg/mL of FU248 for 0, 6 or 24 hr. Total RNAs extracted from the neurons were subjected to reverse transcription. Transcripts for BDNF (open circles), NGF (triangles), NT-3 (squares), and NT-4 (filled circles) were amplified by PCR in the presence of [α-^{32}P]dCTP. Each datum is expressed as a percentage of the expression level of BDNF at 0 hr. Each data bar represents a mean ± SEM (N = 3). Significance was determined using the value at 0 hr for each neurotrophin ($^{**}P < 0.01$).

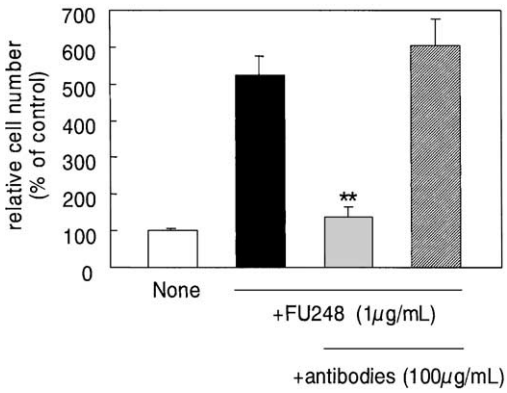

Fig. 5. Neutralization of the neuroprotective effect of FU248 by an anti-BDNF antibody. Primary cortical neurons were cultured in TIP/DF medium for 3 days *in vitro* in the presence of 1 μg/mL of FU248 and either an anti-BDNF antibody (stippled columns) or a control antibody (hatched columns). Surviving neurons were quantified by FDA method. Each data bar represents a mean ± SEM (N = 4). Significance was determined using the value of the solid column ($^{**}P < 0.01$).

4.2. *Effects of FU248 on gene expression profile of neurotrophic factors*

Effects of FU248 on gene expression profile were investigated in an attempt to elucidate cellular and molecular mechanisms underlying the neuroprotective activity of FU248. We especially focused on the gene expression of neurotrophic factors. For this purpose, rat primary cortical neurons were cultured in TIP/DF medium and exposed to 1 μg/mL of FU248 for 0, 6, and 24 hr. RNAs were prepared from the cells, and the expression levels of BDNF, NGF, NT-3, and NT-4 were estimated by semiquantitative RT–PCR. As indicated in Fig. 4, expression of BDNF, NGF, and NT-3 transiently and significantly increased 6 hr after the addition of FU248 and returned to the basal level after 24 hr, whereas expression of NT-4 was not detectable throughout the experimental period. The expression levels of the neurotrophic factors are directly comparable with each other since the radioactivities were normalized to the number of deoxycytidine residues in the PCR products and then to the expression level of S26. The amount of the transcript for BDNF was approximately nine times and sixteen times more abundant than those for NT-3 and NGF, respectively ($t = 6$ hr). These data implied that the contribution of BDNF to the neuronal survival was greater than that of NGF or NT-3. Therefore, the effect of FU248 on the neuronal survival was further investigated using an anti-BDNF antibody. For this purpose, the rat primary cortical neurons cultured in TIP/DF medium were exposed to 1 μg/mL of FU248 for 3 days *in vitro* in the presence of 100 μg/mL of an anti-BDNF antibody, which was raised against recombinant BDNF rather than a synthetic peptide. As clearly demonstrated in Fig. 5, the anti-BDNF antibody almost abolished the neuroprotective effect of FU248. On the other hand, a control antibody did not change the survival rate of the primary cortical neurons at the same concentration. These data strongly suggest that FU248 facilitated survival of rat primary cortical neurons through induction of gene expression of BDNF.

5. Discussion

Primary cortical neurons prepared from the brains of rat embryos (E18) cannot survive without trophic support, and cell death can be induced by serum deprivation. We employed this model for random screening of our library of low molecular weight compounds to develop drugs which inhibit neuronal cell death caused by cerebral ischemia. We found that a new 5-phenylpyrimidine derivative named FU248 inhibited the cell death of rat primary cortical neuron *in vitro* in a dose-dependent manner up to 1 μg/mL (Fig. 2). FU248-dependent survival of the cortical neurons declined at the concentrations over 1 μg/mL probably because of its toxicity (unpublished data). As indicated in Fig. 3, most of the surviving cells had axons and dendrites which were immunoreactive to an anti-MAP-2 antibody. Cytoplasm of the cells was also MAP-2 positive. Weak staining in nuclei of the cells may be caused by nonspecific reaction to the anti-MAP-2 antibody. These morphological and immunohistochemical features indicate that most of the surviving cells were neurons.

We then tried to analyze a possible mechanism underlying the neuroprotective activity of FU248. We especially focused on the effects of FU248 on the gene expression profile of neurotrophic factors by means of semiquantitative RT–PCR analysis. Interestingly, FU248 transiently and significantly induced gene expression of BDNF, a neurotrophin known to protect neurons from death under

various conditions [7–10]. The induction was maximal 6 hr after the addition of FU248, and the expression returned to the basal level after 24 hr. FU248 also transiently induced gene expression of NGF and NT-3 to a lesser extent. On the other hand, NT-4 was not induced at all by FU248 (Fig. 4). Moreover, an anti-BDNF antibody used in the current study abolished the effect of FU248 whereas a control antibody did not show any inhibitory activity. Therefore, it is likely that BDNF induced by FU248 contributed to the survival of rat primary cortical neurons probably by acting in autocrine and/or paracrine fashion [11,12]. We observed that FU248 induced transient increase in intracellular calcium level in a mouse neuroblastoma cell line, Neuro2a (unpublished data). This finding is consistent with the fact that BDNF is one of the immediate early genes expressed in response to increase in intracellular calcium [13–16]. However, it is possible that still other calcium-responsive genes may also have been induced by FU248. Therefore, a possibility that expression of such genes contributed to the survival of rat primary cortical neurons cannot be ruled out.

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