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ORIGINAL CONTRIBUTION

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Eicosapentaenoic acid (EPA) increases cell viability and expression of neurotrophin receptors in retinoic acid and brain-derived neurotrophic factor differentiated SH-SY5Y cells

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Abstract *Background* The n-3 polyunsaturated fatty acid, eicosapentaenoic acid (EPA) has been found to process neuroprotective effects. However, the exact cellular mechanisms are not well understood. Brain-derived neurotrophic factor (BDNF) is one of neurotrophins, which is involved in neuron differentiation, survival, and synaptogenesis. *Aim of the study* In this study, the potential neuroprotective effects of EPA, and its possible effects on BDNF and BDNF receptor expression were investigated in SH-SY5Y cells. *Methods* Both undifferentiated and retinoic acid (RA)-BDNF differentiated SH-SY5Y cells were treated with EPA and/or BDNF. The cell viability was determined by MTT assay. The expression of BDNF receptors, tyrosine kinase receptor B (TrkB) and $p75^{NTR}$ were tested by RT-PCR and Western blotting. *Results* In undifferentiated SH-SY5Y cells, either EPA or BDNF, or both did not affect the cell viability. In RA-BDNF differentiated SH-SY5Y cells, treatment with different doses of EPA (0.01, 0.1, 1.0, 10.0 μM) and BDNF (1 ng/ml) for 24 hours significantly increased the cell viability, while EPA or BDNF alone showed no effect. More importantly, RT-PCR and Western blotting results revealed that 24 hours treatment with EPA (0.01, 0.1, 1.0 μM) significantly increased the full-length TrkB ($TrkB^{TK+}$), but not truncated TrkB ($TrkB^{TK-}$) expression in these cells. An increase in $p75^{NTR}$ expression was also observed with 10.0 μM EPA treatment. Finally, co-incubation with either 100 nM staurosporine, a protein kinase inhibitor, or 500 nM K252a, a tyrosine kinase inhibitor completely abolished the EPA-induced increase in cell viability.
Conclusions Our results indicate that EPA exerts beneficial effects on cell survival through modulating neurotrophin receptor expression.

Key words eisosapentaenoic acid (EPA) – brain-derived neurotrophic factor (BDNF) – tyrosine kinase receptor B (TrkB) – $p75^{NTR}$ – neuroprotection

Introduction

Eicosapentaenoic acid (EPA) is one of essential n-3 polyunsaturated fatty acids (PUFAs) that cannot be synthesized by the human body. Our main dietary sources of EPA are cold-water fish such as wild salmon. Increased intake of EPA has been shown to be beneficial in coronary heart disease [24], and inflammatory disorders such as rheumatoid arthritis

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[20]. The evidence accumulated during the past decade demonstrates that EPA has neuroprotective effects. Initially, epidemiological studies showed that the prevalence of Alzheimer's disease (AD) negatively correlates with fish consumption [9]. Moreover, n-3 PUFAs improved cognitive functions in very old people [15]. A given mixture of n-6 and n-3 fatty acids also improved the life quality of AD patients [36]. Recent studies showed that EPA protected rat hippocampal neurons from lipopolysaccharide (LPS)-induced neurotoxicity both in vitro and in vivo [22]. Furthermore, feeding rats with ethyl-EPA (E-EPA) attenuated IL-1β central administration–induced spatial memory deficit [33]. Supplementing the diet with fish oil (enriched in both docosahexaenoic acid and EPA) decreased the cerebral infarction volume following ischemia and reperfusion (I/R) in rats [6]. However, the cellular mechanisms of the neuroprotective effects of EPA remain unclear.

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family of growth factors, has been shown to modulate survival, differentiation, and activity of neurons [10]. The biological effects of BDNF on neuronal cells are mediated by two specific receptors, tyrosine kinase receptor B (TrkB) and $P75^{NTR}$. The TrkB belongs to the family of tyrosine kinase receptors and binds BDNF in a specific manner. The transcript encoded by the TrkB gene can be alternatively spliced to produce either full-length ($TrkB^{Tk+}$) or truncated ($TrkB^{Tk-}$) isoforms of the receptor [19, 26, 35]. BDNF signaling occurs when $TrkB^{Tk+}$ receptors form homodimers. The $TrkB^{Tk-}$ receptor lacks an intracellular tyrosine kinase domain and can form non-functional homodimers or non-functional heterodimers with $TrkB^{Tk+}$ receptors, thereby inhibiting BDNF signaling [7, 28]. Additionally, $TrkB^{Tk-}$ receptors have been found to be able to mediate intracellular signal transduction upon BDNF-mediated activation [2, 31]. The $p75^{NTR}$ receptor does not show any binding preference for the different neurotropins [4] and has been found to be involved in the regulation of neuronal survival due to its ability to induce apoptosis and modify the function of Trk receptors [18].

Continuously dividing cell lines such as neuroblastoma SH-SY5Y have been widely used as a model to study neurodegenerative disorders such as Alzheimer's [11, 21, 30] and Parkinson's diseases [32]. SH-SY5Ys differentiate into cells that are biologically, ultrastructurally, and electrophysiologically similar to neurons [1] after being treated with different agents such as neurotrophic factors [16], retinoic acid (RA), phorbol ester [29], or staurosporine [13]. RA causes the differentiation of SH-SY5Y cells and mediates biological responsiveness of the cells to the TrkB ligands BDNF and NT-3 by stimulating the expression of TrkB receptors [16]. Sequential differentiation of SH-SY5Ys with RA and BDNF gives rise to fully differentiated, human neuron-like cells [8].

In the present study, we hypothesized that the previous reported neuroprotective effects of EPA may be associated with the regulation of BDNF and/or BDNF receptor expression. Therefore, in the present study, we decided to examine the changes in both cell viability and expression of BDNF and BDNF receptors in RA-BDNF differentiated SH-SY5Y cells following exposure to EPA. Our results showed that EPA significantly increased cell viability in RA-BDNF differentiated, but not undifferentiated SH-SY5Ys. Furthermore, EPA significantly up-regulated the expression of $TrkB^{Tk+}$ and $p75^{NTR}$, but not $TrkB^{Tk-}$ and BDNF in RA-BDNF differentiated SH-SY5Ys. Co-incubation with STS, a protein kinase inhibitor, or K252a, a tyrosine kinase inhibitor completely abolished the EPA-induced cell viability increase. These data indicate that EPA mediates beneficial effects on cell survival through up-regulation of neurotrophin receptor expression. Further clarifications both in vitro and in vivo are warranted.

Materials and methods

Cell culture and differentiation

The human neuroblastoma cell line SH-SY5Y was kindly provided by Dr. M. Mayne at National Research Council Canada Institute for Nutriscience and Health (NRC-INH). The cells were cultured in DME/F12 culture medium supplemented with 10% FCS, 1% penicillin–streptomycin, 1% MEM, 1 mM sodium pyruvate, 1.5 g/l sodium bicarbonate on 75 cm^2 flasks. Cells were seeded at an initial density of 3×10^5 cells/cm^2 in culture plates (BD Biosciences) previously coated with 1 μg/ml laminin. Twenty-four hours after seeding, cells were sequentially differentiated with RA and BDNF as reported previously [8]. Briefly, all-*trans*-RA (Sigma) was added the day after plating at a final concentration of 10 μM in DME/F 12 with 10% FCS. After 6 days in the presence of RA, cells were washed two times with DME/F 12 (serum free) and incubated with 1 ng/ml BDNF (Invitrogen) in DME/F 12 (serum free) for 3 days.

Experimental treatments for cells

Both undifferentiated and RA-BDNF differentiated SH-SY5Y cells were incubated for 24 hours with different concentrations of EPA (0.01, 0.1, 1.0, 10 μM) either in the absence or in the presence of 1 ng/ml BDNF. RA-BDNF differentiated SH-SY5Ys were also treated with different concentrations of EPA (0.01, 0.1, 1.0, 10 μM) and 1 ng/ml BDNF in the presence of either 100 nM STS (Sigma) or 500 nM K252a (Sigma).

MTT assay for cell viability

The mitochondrial dehydrogenase activity that cleaves the tetrazolium salt 3-[4,5-Dimethylthiazol]-2,5-diphenyltetrazolium (MTT) to the formazan product was used to determine cell viability in a colorimetric assay. Briefly, cells were seeded in 96-well plates and grown to 80% confluence in culture medium. The medium was replaced by medium containing various concentrations of EPA and/or BDNF. MTT (Sigma) was added to the culture media at a final concentration of 0.25 mg/ml and incubated for another 4 hours at 37°C. After the medium was removed, cells and dye crystals were solubilized with 200 μL DMSO and absorbance was measured at 570 nm by a microplate assay reader (Molecular Devices). The results were expressed as percentage of control culture viability.

Reverse transcription-polymerase chain reaction (RT-PCR)

The total RNA was purified from cells by using the Trizol reagent according to the manufacturer's instructions (Invitrogen). To remove contaminating genomic DNA, samples were treated with Rnase-free DNase I (Fermentas). Reverse transcription (RT) was carried out in 20 µl volume containing 1 µg of total RNA (Fermentas First Strand cDNA Synthesis Kit). The conditions for Polymerase chain reaction (PCR) were as follows. One microliter of cDNA products were added in the presence of 0.4 µM primers, 200 µM deoxy-NTPs, 2.5 U *Taq* DNA polymerase (Qiagen), and 1 × *Qiagen* PCR buffer that contained 1.5 mM $MgCl_2$. After denaturation at 94°C for 3 minutes, 30–40 cycles of amplication (94°C for 30 seconds, 55–66°C for 30 seconds, and 72°C for 30 seconds) followed by a final extension at 72°C for 7 minutes were performed. The reaction products were separated electrophoretically on a 1.5% ethidiumbromide-stained agarose gel and quantified by the gel imaging system (Scion Image, Scion Corporation, MD). Experiments were conducted in which cDNA samples were amplified with different cycle numbers for TrkB, $p75^{NTR}$, and β-actin primers to assure that DNA bands after amplification were detected within the linear part of the amplifying curves (30 cycles for $TrkB^{Tk+}$, 30 cycles for $TrkB^{Tk-}$, 40 cycles for $p75^{NTR}$, 35 cycles for BDNF, 30 cycles for β-actin). The following sets of primers were used: **β-actin** forward 5′-AGGGTACATGGTGGTGCCGCCA GAC-3′, reverse 5′-AGGGTACATGGTGGTGCCGC-C AGAC-3′, **BDNF** forward 5′-TCCACCAGGTGAGAA GAGTG-3′, reverse 5′-ACTTGA-CTACTGAGCATC AC-3′, **$TrkB^{Tk+}$** forward 5′-CATGTTACCAATCACA CGGAGTACC-3′, reverse 5′-GGATCTTGGTCATTC CAATAATGAC-3′, **$TrkB^{Tk-}$** forward 5′-CATGT-TAC CAATCACACGGAGTA-3′, reverse 5′-CCATCCAGTG GGATC-TTATGAAA-3′, **$p75^{NTR}$** forward 5′-CTGGA CAGCGTGACGTTCTCC-3′, reverse 5′-CTGCCAC CG TGC-TGGCTATGA-3′. The band intensities were correlated to those of β-actin, which allows for an estimation of the integrity of the RNA (expression levels of target mRNA were normalized to β-actin).

Western blotting analysis

Cells were washed twice in PBS buffer, and then centrifuged at 2,000 rpm for 10 minutes. The cell pellet was lysed in buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, supplemented with a cocktail of protease inhibitors (Roche Diagnostics). Cell lysates were centrifuged at 13,000 *g* at 4°C for 10 minutes. The supernatants were used for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) after quantification of the amount of total protein by using BCA Protein Assay kit (Pierce). Aliquots containing 100 µg protein were loaded and separated on 6% (for $TrkB^{Tk+}$ and $TrkB^{Tk-}$) and 7.5% (for $p75^{NTR}$ and β-actin) polyacrylamide gels at 100 V for 70 minutes. After separated on SDS–PAGE, proteins were transferred on to nitrocellulose membrane (Hybond, Amersham Biosciences, Amersham, UK) in transfer buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol] for 2 hours at 0.4 A at 10°C and blocked for 1 hours at room temperature in Tris-buffered saline–Tween-20 (TBS-T) [50 mM Tris, pH 8.0, 133 mM NaCl, 0.05% (v/v) Tween-20] with 5% (w/v) non-fat milk powder (Carnation, Nestle). The blot was incubated with polyclonal anti-TrkB antibody (1:250), polyclonal anti-$p75^{NTR}$ antibody (1:100) and polyclonal anti-β-actin antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C in TBS-T with 1% (w/v) non-fat milk powder. Then they were incubated in horseradish peroxidase (HRP)-conjugated donkey anti-goat secondary antibodies (1:5,000) (Santa Cruz Biotechnology) in TBS-T with 1% non-fat milk powder for 1 hour at room temperature. All antibodies were from Santa Cruz Biotechnology. Immunoreactive bands were detected with the Super-Signal West Pico Chemiluminescence Substrate (Pierce) and a CCD camera (Bio-Rad).

Statistical analysis

Data for mRNA expression, protein expression and cell viability were expressed as mean ± standard error of mean (SEM) and analyzed using GraphPad Prism 4.0 with one-way analysis of variance (ANOVA) followed by Tukey post-hoc test. Differences

were considered significant at $P < 0.05$. Protein expressions by measured Western blotting were expressed as mean ± SEM (n = 3–4).

Results

Sequential treatment with RA and BDNF differentiated SH-SY5Y cells into fully differentiated human neuron-like cells

As shown in Fig. 1, undifferentiated SH-SY5Y cells showed round features (Fig. 1a) with few short processes as photographed by phase contrast microscope. Treatment with 10 μM RA for 6 days induced modest neuronal differentiation resulting in smaller cell bodies and a more neuron-like phenotype with loss of the round morphology and signs of neurite outgrowth (Fig. 1b). After sequentially treated with 1 ng/ml BDNF for another 3 days, the cells displayed a mature neuronal morphology with more neurite extensions connecting the cells (Fig. 1c), which was morphologically not essentially different from previous reports using 50-fold higher doses of BDNF [8].

Effects of EPA on cell viability in both RA-BDNF differentiated SH-SY5Y cells and undifferentiated SH-SY5Y cells

We next investigated whether EPA had an effect on cell viability either in RA-BDNF differentiated or in undifferentiated SH-SY5Y cells. Both RA-BDNF differentiated and undifferentiated SH-SY5Ys were incubated with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM) either in the absence or in the presence of 1 ng/ml BDNF for 24 hours. The cell viability was determined by MTT assay. The concentrations of EPA used were determined by preliminary experiments (data not shown). As shown in Fig. 1d, although 24 hours incubation with either EPA or BDNF alone did not affect cell viability in RA-BDNF differentiated SH-SY5Ys, co-incubation with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM) and 1 ng/ml BDNF significantly increased the cell viability

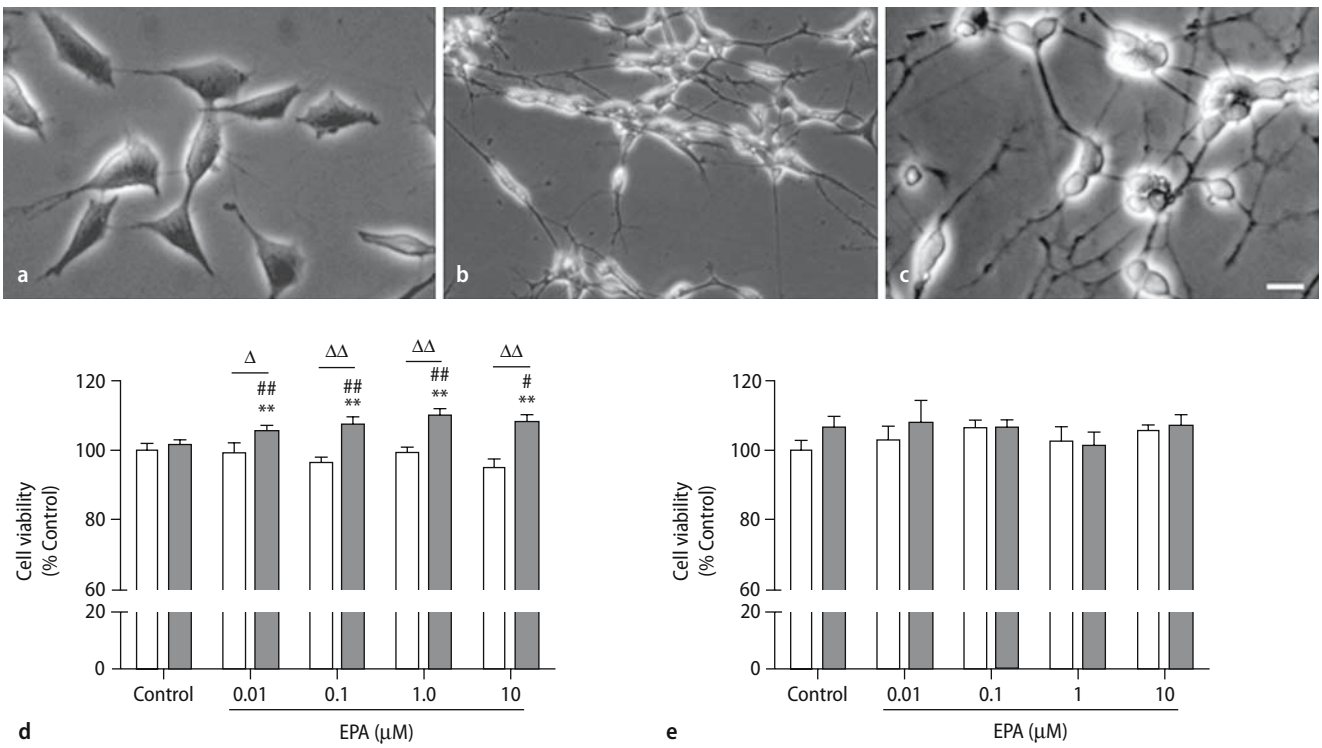

Fig. 1 Effects of sequential treatment with RA and BDNF on cell differentiation of SH-SY5Y cells and effects of 24 hours treatment with EPA on cell viability of these cells. Neuroblastoma cell line SH-SY5Y cells (**a**) were sequentially incubated with all-*trans*-RA (10 μM) for 6 days (**b**), followed by BDNF (1 ng/ml) for 3 days (**c**). Pictures were taken under a phase-contrast microscope. Original magnification ×200 for **a**–**c**. *Bar* 30 μm. Both RA-BDNF differentiated (**d**) and undifferentiated (**e**) SH-SY5Y cells were treated with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM) either in the absence or in the presence (*partially filled square*) of BDNF (1 ng/ml) for 24 hours. Cell viability was determined by MTT assay. Data are expressed as mean ± SEM. $^{**}P < 0.01$ versus control without BDNF; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control with BDNF; $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ (ANOVA, n = 6). Data are representative of six independent experiments

of these cells either compared with the control without BDNF ($P < 0.01$, respectively) or compared with the control with BDNF ($P < 0.01$ at 0.01, 0.1 and 1.0 μM of EPA, respectively; $P < 0.05$ at 10.0 μM of EPA). In contrast, in undifferentiated SH-SY5Y cells, 24 hours incubation with EPA either in the absence or in the presence of BDNF showed no effect on cell viability (Fig. 1e).

Effects of EPA on the expression of BDNF and BDNF receptors in RA-BDNF differentiated SH-SY5Y cells

We then determined whether EPA affected the mRNA expression of both BDNF and its receptors in RA-BDNF differentiated SH-SY5Y cells. RA-BDNF differentiated SH-SY5Ys were incubated with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM) (in the absence of BDNF) for 24 hours. The mRNA expression of BDNF, $TrkB^{Tk+}$, $TrkB^{Tk-}$, and $p75^{NTR}$ were semi-quantified by RT-PCR. Six-day differentiation with 10 μM RA significantly increased the mRNA expression of BDNF (Fig.2a, b; $P < 0.01$), $TrkB^{Tk+}$ (Fig. 2a, c; $P < 0.01$), $TrkB^{Tk-}$ (Fig. 2a, d; $P < 0.01$), and $p75^{NTR}$ (Fig. 2a, e; $P < 0.05$) compared to undifferentiated SH-SY5Ys. Sequential differentiation with 1 ng/ml BDNF for another 3 days significantly decreased the mRNA expression of BDNF (Fig. 2a, b; $P < 0.01$), $TrkB^{Tk+}$ (Fig. 2a, c; $P < 0.01$), but not $TrkB^{Tk-}$ (Fig. 2a, d; $P > 0.05$) and $p75^{NTR}$ (Fig. 2a, e; $P > 0.05$) as compared with the RA-differentiated cells. However, the $TrkB^{Tk+}$ (Fig. 2a, c; $P < 0.01$) and $TrkB^{Tk-}$ (Fig. 2a, d; $P < 0.01$) mRNA expression was still significantly higher than the undifferentiated SH-SY5Ys. In RA-BDNF differentiated SH-SY5Ys, although 24 hours treatment with EPA showed no effects on both BDNF (Fig. 2a) and $TrkB^{Tk-}$ (Fig.2a, d) mRNA expression, EPA significantly upregulated the $TrkB^{Tk+}$ mRNA expression at different concentrations (Fig. 2a, c, $P < 0.05$ for 0.01 μM and $P < 0.01$ for 0.1, 1.0, 10.0 μM, respectively). Additionally, a significant increase in $p75^{NTR}$ mRNA expression by 10 μM EPA treatment compared to the RA-BDNF differentiated SH-SY5Ys (Fig. 2a, e; $P < 0.01$), but not by 0.01, 0.1, and 1.0 μM EPA treatment was found (Fig. 2a, e; $P > 0.05$, respectively). β-actin was amplified as internal control.

We next tested the effects of EPA on $TrkB^{Tk+}$, $TrkB^{Tk-}$, and $p75^{NTR}$ expression in RA-BDNF differentiated SH-SY5Ys by Western blotting. Due to the different baseline band intensity (as measured by ImageJ, we calculated treatment groups as percentage of control within each experiment). Furthermore, due to the limited number of Western experiments performed, statistics were not calculated. Nonetheless, the data showed patterns that are somewhat consistent with mRNA expression. As shown in Fig. 3, six-day differentiation with 10 μM RA increased the expression of $TrkB^{Tk+}$ (145 kDa) (Fig. 3a, b) and $TrkB^{Tk-}$ (95 kDa) (Fig. 3a, c), but not $p75^{NTR}$ (75 kDa) (Fig. 3a, d) compared to undifferentiated cells. Sequential differentiation with 1 ng/ml BDNF for another 3 days decreased the expression of $TrkB^{Tk+}$, but not $TrkB^{Tk-}$ and $p75^{NTR}$ (Fig. 3a–d) as compared with the RA-differentiated SH-SY5Ys. Importantly, 24 hours treatment with different concentrations of EPA (0.01, 0.1, 1.0 μM) increased the expression of $TrkB^{Tk+}$ (Fig. 3a, b), but not $TrkB^{Tk-}$ (Fig. 3a, c). However, a decrease of $TrkB^{Tk+}$ was found at 10 μM of EPA. In addition, although 24 hours treatment with 0.01, 0.1, and 1.0 μM of EPA showed no effects on $p75^{NTR}$ expression, an increase in $p75^{NTR}$ expression was found with 10 μM EPA treatment (Fig. 3a, d). β-actin (43 kDa) was used to confirm equal loading of proteins (Fig. 3a).

Protein kinase inhibitors abolished EPA-induced cell viability increase in RA-BDNF differentiated SH-SY5Y cells

We then investigated whether protein kinase inhibitors had effects on EPA-induced cell viability increase in RA-BDNF differentiated SH-SY5Ys. The cells were incubated with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM) and 1 ng/ml BDNF both in the absence and in the presence of STS (100 nM), a protein kinase inhibitor, or K252a (500 nM), a tyrosine kinase inhibitor for 24 hours. The cell viability was determined by MTT assay. The concentrations of STS and K252a used were determined by preliminary experiments (data not shown). Either 100 nM STS or 500 nM K252a alone showed no effects on cell viability in these cells (Fig. 4a, b). While co-incubation with BDNF and different concentrations of EPA significantly increased cell viability (Fig. 4a, b), adding 100 nM STS to the culture system completely abolished this effect (Fig. 4a). Similar results were found for adding 500 nM K252a to the culture system (Fig. 4b).

Discussion

In the present study, we have demonstrated that EPA increases cell viability in RA-BDNF differentiated, but not undifferentiated SH-SY5Y cells. This effect is dependent on the co-existence of BDNF in the culture media. Although no effect of EPA on BDNF mRNA expression in these cells is shown, up-regulations in mRNA expression of BDNF receptors, $TrkB^{Tk+}$ and $p75^{NTR}$, but not $TrkB^{Tk-}$ are found. Protein

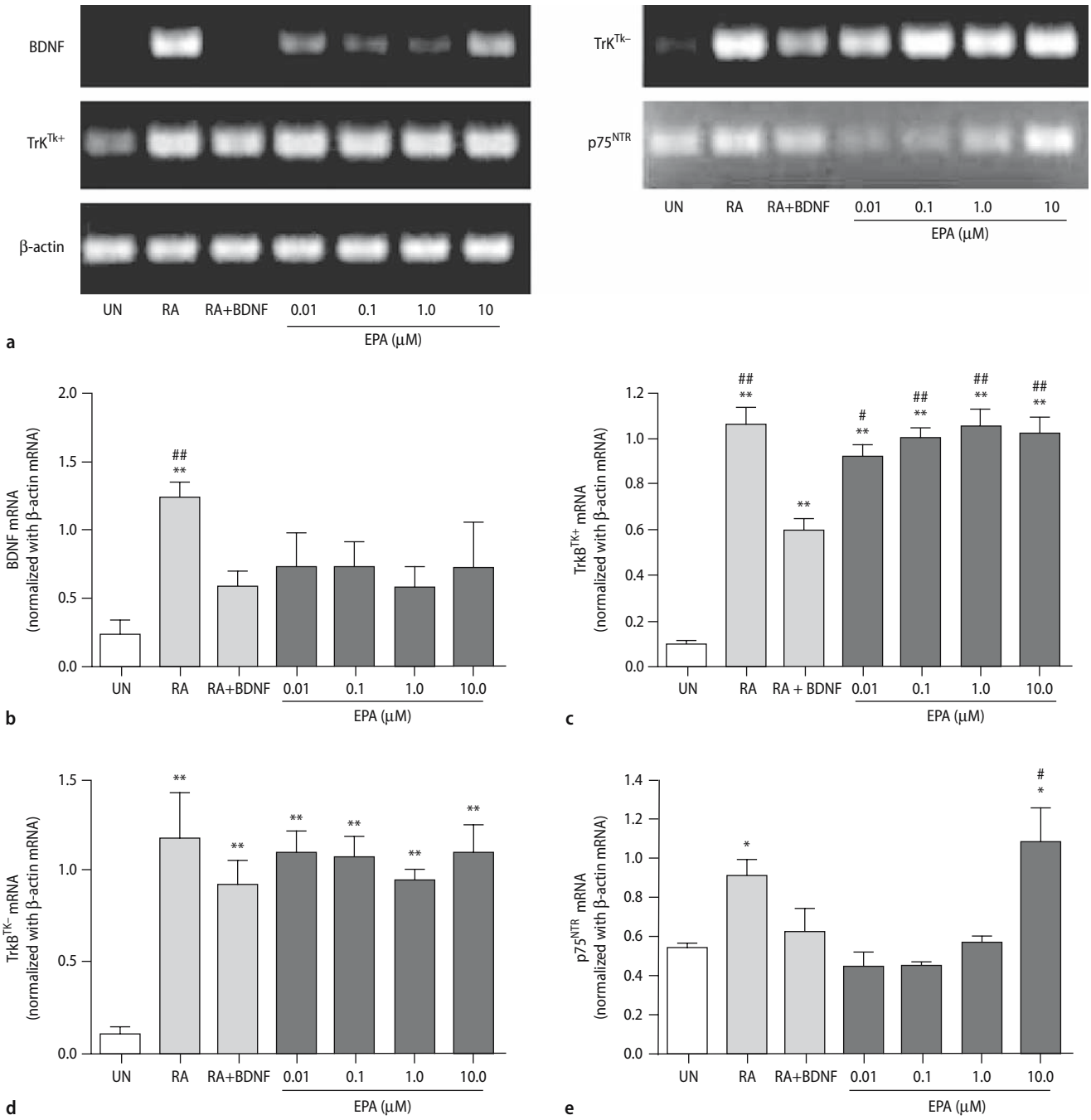

Fig. 2 The mRNA expression of BDNF, TrkB^{Tk+}, TrkB^{Tk-}, and p75^{NTR} in SH-SY5Y cells in response to differentiation and subsequent treatment with EPA for 24 hours. Semi-quantitative analyses of BDNF, TrkB^{Tk+}, TrkB^{Tk-}, and p75^{NTR} mRNAs by RT-PCR, with β-actin as the internal control. Ethidium bromide-stained gel pictures are representative of experimental results obtained from six independent experiments (**a**). BDNF (**b**), TrkB^{Tk+} (**c**), TrKB^{Tk-} (**d**), and p75^{NTR} (**e**) mRNA expression is normalized on the basis of β-actin expression. Data are expressed as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ versus UN; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus RA+BDNF

expressions measured by Western blotting are in line with these findings. Finally, either protein kinase inhibitor STS, or tyrosine kinase inhibitor K252a completely abolishes the EPA-induced up-regulation of cell viability in RA-BDNF differentiated SH-SY5Ys.

Sequential exposure of neuroblastoma cell line SH-SY5Y to RA and BDNF yielded homogeneous populations of human neuron-like cells, which presented many characteristics of primary neuron cultures and did not contain glial cells [8]. To date, this model has

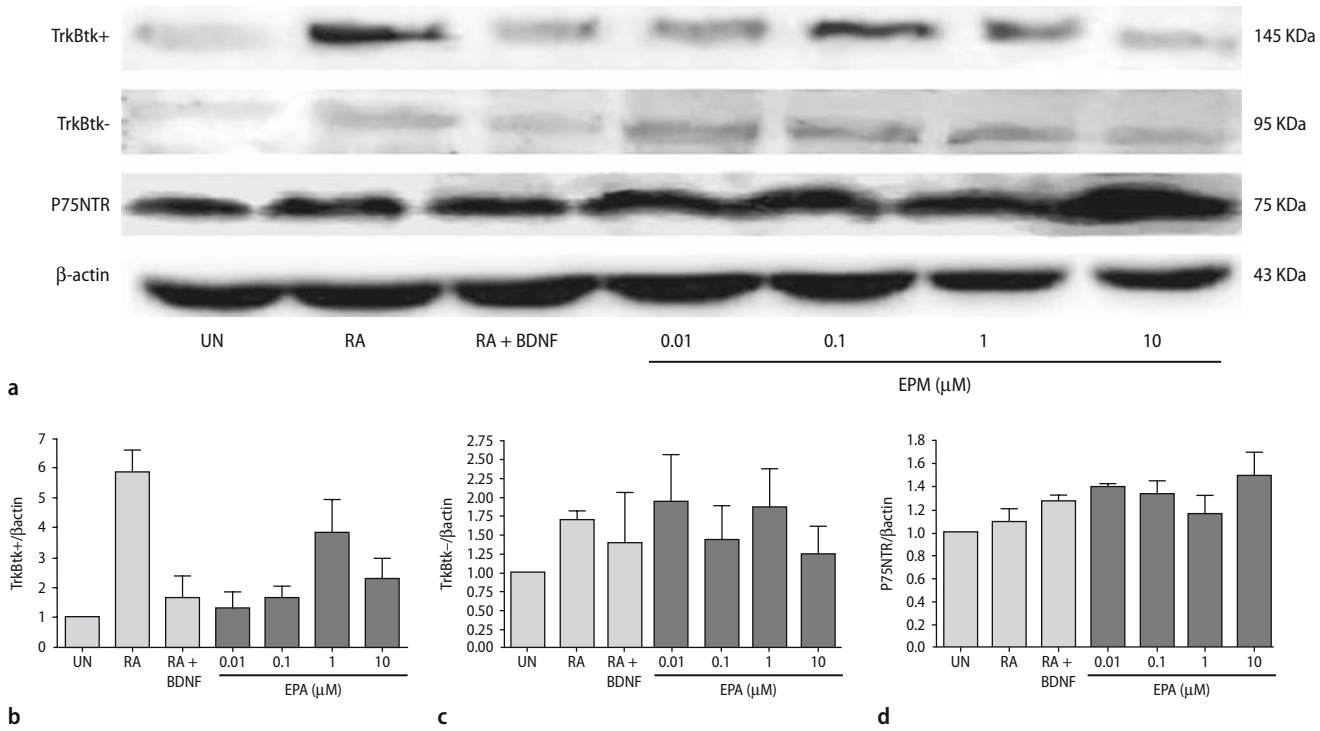

Fig. 3 The $TrkB^{Tk+}$, $TrkB^{Tk-}$, and $p75^{NTR}$ expression in SH-SY5Y cells in response to differentiation and subsequent treatment with EPA for 24 hours. Representative western blot of $TrkB^{Tk+}$, $TrkB^{Tk-}$, $p75^{NTR}$ and β-actin expression are shown. $TrkB^{Tk+}$ (**b**), $TrKB^{Tk-}$ (**c**), and $p75^{NTR}$ (**d**) expression is normalized to β-actin expression

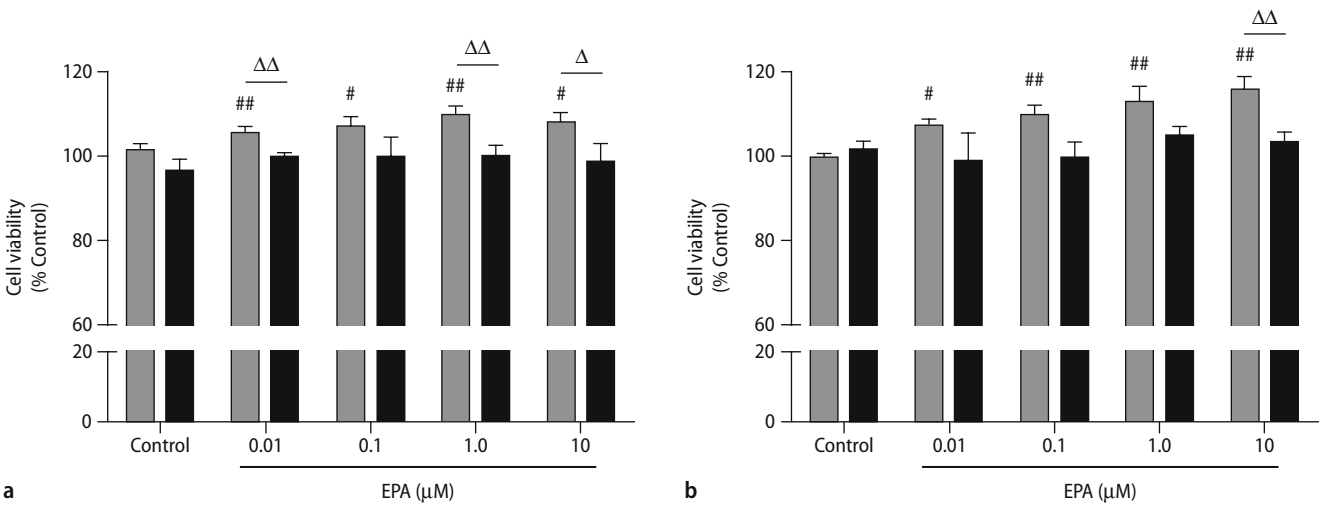

Fig. 4 Effects of co-incubation of staurosporine (*STS*) or K252a with EPA on cell viability in RA-BDNF differentiated SH-SY5Y cells. The cells were treated with different concentrations of EPA (0.01, 0.1, 1.0, 10.0 μM), 1 ng/ml BDNF, either in the absence (*crossed bar*) or in the presence (*filled bar*) of 100 nM STS (**a**) or 500 nM K252a (**b**) for 24 hours. Cell viability was determined by MTT assay. Data are expressed as mean ± SEM. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus control without STS (**a**) or K252a (**b**); $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ (ANOVA, $n = 6$). Data are representative of three independent experiments

been widely used in experimental neurological studies [12, 14]. In our study, the morphological differentiation of SH-SY5Ys is confirmed by phase-contrast microscopy. Furthermore, an EPA-induced up-regulation in cell viability is found in these cells, indicating EPA is able to benefit cell survival in vitro. This result is in parallel with our previous in vivo studies, which demonstrate that diets enriched in EPA enhanced spatial learning and memory and reduced anxiety-related behavior in rats [33, 34]. Results from

independent group have also demonstrated that EPA is able to protect rat hippocampal neurons through its ability to inhibit the activation of c-Jun N-terminal kinase (JNK) [22]. The EPA-induced anti-apoptotic effect in rat brain is also reported [23]. In line with these findings, our data indicate that EPA is able to promote neuronal survival.

Interestingly, in the present study, we find that EPA only increases cell viability in RA-BDNF differentiated SH-SY5Y cells, and the presence of BDNF in the culture system is another prerequisite. Previous studies have demonstrated that RA differentiation of SH-SY5Ys mediates biological responsiveness of the cells to BDNF by stimulating the expression of TrkB receptors [16], while further treatment in these cells with BDNF down-regulates TrkB surface levels [10]. This gives us a clue that the neuroprotective effects of EPA may be mediated through its modulation on neurotrophin and/or neurotrophin receptors. Therefore, we next investigated the effects of EPA on mRNA expression of BDNF, $TrkB^{Tk+}$, $TrkB^{Tk-}$, and $p75^{NTR}$. Our results confirm the previous reports that 6 days RA (10 μM) differentiation upregulates both $TrkB^{Tk+}$ and $TrkB^{Tk-}$ mRNA expression [16]. Furthermore, upregulations of both BDNF and $p75^{NTR}$ mRNA expression are found in the present study. The consequential differentiation with BDNF down-regulates both mRNA expression of $TrkB^{Tk+}$ and BDNF, but not $TrkB^{Tk-}$ and $p75^{NTR}$ in these cells. Importantly, in RA-BDNF differentiated SH-SY5Ys, although no effect of 24 hours EPA treatment on BDNF and $TrkB^{Tk-}$ mRNA expression is found, we do find that EPA is able to increase the mRNA expression of $TrkB^{Tk+}$ and $p75^{NTR}$. These effects are further somewhat confirmed at the protein level with Western blotting (see Fig. 3), although a downregulation of $TrkB^{Tk+}$ was noted at 10 μM EPA treatment. Also, 6 days differentiation with RA did not induce a clear upregulation of P75NTR. Nonetheless, these results taken together thus indicate that EPA is able to modulate the cellular expression of neurotrophin receptors, which may be related to its neuroprotective effects.

In order to further confirm the above observation, we added STS, a protein kinase inhibitor or K252a, a tyrosine kinase inhibitor into our cell culture system to pharmacologically block the tyrosine kinase activity. In the presence of BDNF, either 100 nM STS or 500 nM K252a alone does not influence the cell viability of RA-BDNF differentiated SH-SY5Ys. However, when co-incubated with EPA, either 100 nM STS or 500 nM K252a completely abolishes the EPA-induced cell viability increases in these cells. These results indicate that the tyrosine kinase, which is the active intracellular domain of $TrkB^{Tk+}$, contributes to the EPA-induced biological effects in these cells. Therefore, the EPA-induced beneficial effects on cell survival and up-regulation of the $TrkB^{Tk+}$ expression can be functionally linked; EPA may be able to promote neuronal survival through modulation of neurotrophin receptor expression.

The $TrkB^{Tk+}$-mediated BDNF signaling can be modulated by the low affinity receptor $p75^{NTR}$ [3, 5]. It has been reported that activation of $p75^{NTR}$ induces apoptosis in various cell types in nervous and non-nervous systems [25], and this effect is predominant when Trk receptors are inactive or absent [27]. However, when co-expressed with Trks, $p75^{NTR}$ mediates cell survival rather than apoptosis [17]. In the current study, although no effect of 0.01, 0.1, and 1.0 μM of EPA on $p75^{NTR}$ expression is found, we do find that $p75^{NTR}$ expression is upregulated by incubation with 10 μM EPA. Although the biological consequence of this EPA-induced $p75^{NTR}$ upregulation needs to be further clarified, we suggest that it may be also functionally relevant to the neuroprotective effects of EPA.

The modulation of $TrkB^{Tk+}$-mediated BDNF signaling by the truncated TrkB isoform $TrkB^{Tk-}$ is also reported [19, 26]. Haapasalo et al. [10] found that the expression of $TrkB^{Tk+}$ on the cell surface was reduced when coexpressed with $TrkB^{Tk-}$. The $TrkB^{Tk-}$ receptor-mediated intracellular signal transduction upon BDNF-activation has also been reported [2, 31]. In the current study, we do not find any effects of EPA on $TrkB^{Tk-}$ expression in RA-BDNF differentiated SH-SY5Ys, indicating that $TrkB^{Tk-}$ is not involved in the EPA-mediated biological effects. However, the regulation of TrkB surface expression is tightly controlled by complex mechanisms in active neurons [10]. Additionally, the $TrkB^{Tk-}$ can form non-functional dimers with $TrkB^{Tk+}$ receptors, thereby inhibiting BDNF signaling [7, 28]. Whether $TrkB^{Tk-}$ plays a role in EPA-induced beneficial effects in cell survival needs to be further clarified as well. Furthermore, it needs to be determined how the downregulation of $TrkB^{Tk+}$ protein by EPA 10 μM may relate to cell viability. As shown in Fig. 2, EPA still had positive effects on cell viability, even though there was an apparent downregulation of the receptor (Fig. 3a, b).

In summary, our data demonstrate that EPA increases cell viability in RA-BDNF differentiated SH-SY5Y cells, which is accompanied by up-regulation of neurotrophin receptors, namely $TrkB^{Tk+}$ and $p75^{NTR}$, but not $TrkB^{Tk-}$. This effect is completely abolished by co-incubation with either STS or K252a. These results indicate that EPA is able to exert beneficial effects on neuronal survival by modulating gene expression, in particular, the neurotrophin receptor expression. Although the downregulation of $TrkB^{Tk+}$ protein does seem to be contradictional in these findings, the upregulation of $TrkB^{Tk+}$ protein by lower doses of EPA as well as the abolishing effects of

the tyrosine kinase inhibitors do clearly appear to suggest that EPA mediates its effects of SH-SY5Y cell viability through these receptors. Further experiments conducted both in vitro by treating primary neurons with EPA and, in vivo by feeding animals with E-EPA enriched diets in order to clarify these findings further are warranted.

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