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The estrogen receptor β-PI3K/Akt pathway mediates the cytoprotective effects of tocotrienol in a cellular Parkinson's disease model

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ABSTRACT

Tocotrienols (T3s) are members of the vitamin E family, have antioxidant properties, and are promising candidates for neuroprotection in the pathogenesis of neurodegenerative disorders such as Parkinson's disease (PD). However, whether their antioxidant capacities are required for their cytoprotective activity remains unclear. In this regard, the antioxidant-independent cytoprotective activity of T3s has received considerable attention. Here, we investigated the signaling pathways that are induced during T3-dependent cytoprotection of human neuroblastoma SH-SY5Y cells, as these cells are used to model certain elements of PD. T3s were cytoprotective against 1-methyl-4-phenylpyridinium ion (MPP^+) and other PD-related toxicities. γT3 and δT3 treatments led to marked activation of the PI3K/Akt signaling pathway. Furthermore, we identified estrogen receptor (ER) β as an upstream mediator of PI3K/Akt signaling following γT3/δT3 stimulation. Highly purified γT3/δT3 bound to ERβ directly in vitro, and knockdown of ERβ in SH-SY5Y cells abrogated both γT3/δT3-dependent cytoprotection and Akt phosphorylation. Since membrane-bound ERβ was important for the signal-related cytoprotective effects of γT3/δT3, we investigated receptor-mediated caveola formation as a candidate for the early events of signal transduction. Knockdown of caveolin-1 and/or caveolin-2 prevented the cytoprotective effects of γT3/δT3, but did not affect Akt phosphorylation. This finding suggests that T3s and, in particular, γT3/δT3, exhibit not only antioxidant effects but also a receptor signal-mediated protective action following ERβ/PI3K/Akt signaling. Furthermore, receptor-mediated caveola formation is an important event during the early steps following T3 treatment.

1. Introduction

Vitamin E is a fat-soluble vitamin and is well known as an antioxidant that is present in the cell membrane and protects cells and organs against oxidative stress. There are 8 vitamin E derivatives: α-, β-, γ-, and δ-tocopherol (αTOC, βTOC, γTOC, and δTOC, respectively) and α-, β-, γ-, and δ-tocotrienol (αT3, βT3, γT3, and δT3, respectively) [1,2]. These classes can be differentiated by an unsaturated side chain with three double bonds in the farnesyl isoprenoid tail of TOCs and an isoprenoid tail with 3 double bonds in T3s [1,2]. Furthermore, differences between αT3, βT3, γT3, and δT3 are characterized by the number and location of methyl groups on the chromanol ring. T3s are found only in a few vegetable fats such as palm oil and rice bran oil [3,4]. Numerous reports have suggested that all types of vitamin E scavenge reactive oxygen species and protect cells and organs against oxidative stress as their common potent characteristic [5–7]. Furthermore, recent epidemiological and clinical studies suggest that vitamin E is effective in oxidative stress-related neurodegenerative disorders such as Parkinson's disease (PD) [8–10], Alzheimer's disease [11], and amyotrophic lateral sclerosis [12]. However, other clinical studies have failed to confirm the efficacy of vitamin E members, especially αTOC, in the treatment of these diseases [13–15]. Additionally, the detailed molecular mechanisms by which vitamin E is neuroprotective have not been fully elucidated. Recently, there has been significant interest in the protective mechanisms of vitamin E that are independent of its antioxidative properties; this includes its induction of receptor-mediated signal transduction [2].

PD is a neuropathological disorder involving the degeneration of dopaminergic neurons in the substantia nigra, with the subsequent loss of their terminals in the striatum [16]. The ensuing loss of dopamine causes most of the debilitating motor disturbances associated with PD. Although current PD medications treat the symptoms of the disease without halting or retarding the degeneration of dopaminergic neurons, there has been considerable interest recently in neuroprotection as a

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therapeutic strategy for PD [17,18]. Therefore, several drugs have been proposed as candidate neuroprotective agents for PD [19–21]. Oxidative stress contributes, at least in part, to the pathogenic cascade leading to dopaminergic neuronal degeneration in PD [16,19]. Therefore, antioxidants are potential candidates for neuroprotective therapy against PD. However, antioxidative therapy for PD using exogenous antioxidants has not been successful in the clinical setting thus far [22]. A large-scale study for the effects of a monoamine oxidase inhibitor (deprenyl) and αTOC on the progression of early PD, referred to as the "DATATOP" study, confirmed the efficacy of deprenyl but not of αTOC [20]. However, interestingly, dietary epidemiological studies suggest that sufficient intake of vitamin E may prevent the onset of PD [8,9]. This finding suggests that other non-oxidative cytoprotective effects of vitamin E in addition to those provided by αTOC may be beneficial for PD prevention. In this sense, T3s may represent promising candidates for PD prevention.

We previously reported that deprenyl and caffeine exhibit cyto- and neuroprotective effects via PI3K-dependent signals in a cell model of PD using SH-SY5Y cells [19,23]. These neuroprotective agents can interact with several receptors on the plasma membrane and in doing so activate PI3K/Akt signaling [19]. Although T3s are known to activate PI3K/Akt signaling in cerebral cortical neurons [24], direct target molecules of T3s upstream of PI3K/Akt have not yet been identified. We hypothesized that T3s also have cytoprotective effects against PD-related toxins, and that this depends not only upon an antioxidative effect, but also upon receptor-mediated signal transduction via PI3K/Akt-related signals. The aim of this study was to identify the target molecule of T3s that mediates PI3K/Akt-dependent neuroprotection, and to clarify the early steps of membrane trafficking involved in the process.

2. Material and methods

2.1. Chemicals and antibodies

αT3, βT3, γT3, and δT3 were obtained from Eisai Food Chemical Inc. (Japan, Tokyo). 1-Methyl-4-phenylpyridinium ion (MPP^+), the adenosine A_{2A} receptor antagonist 8-(3-chlorostyryl) caffeine, and the D2 dopamine receptor antagonist droperidol were purchased from Sigma. MG132 and the Trk neurotrophin receptor antagonist K252a were purchased from Calbiochem (La Jolla, CA, USA). All-*trans*-retinoic acid (RA), thapsigargin, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrasolium bromide (MTT), the estrogen receptor antagonist tamoxifen, 17β-estradiol (E2), and Hoechst 33342 were obtained from Wako (Osaka, Japan). 2,4,6,7-^{3}H (N)-Estradiol (^{3}H-E2) was obtained from Perkin Elmer Life and Analytical Sciences (Waltham, MA, USA). The PI3K inhibitor LY294002 and wortmannin were purchased from Alomone Labs (Jerusalem, Israel). The Erk MAPK inhibitor PD98059, the p38 MAPK inhibitor SB203580, and the JNK inhibitor SP600125 were purchased from Alexis (San Diego, CA, USA). Full-length estrogen receptors α (ERα) and β (ERβ) were obtained from Invitrogen (Carlsbad, CA, USA). The following antibodies were used in this study. Anti-phospho-Erk (E-4), anti-Erk1 (K-23), anti-phospho-p38 (Tyr182-R), anti-p38 (H-147), anti-phospho-JNK (Thr183/Tyr185-R), anti-JNK (D-2), anti-Akt1/2 (H-136), anti-ERα (H226), anti-caveoline 1 (7C8), and anti-caveoline 3 (A-3) were obtained from Santa Cruz Biotechnology. Anti-phospho-Akt (Ser473), anti-β-actin, and anti-ERβ were purchased from Cell Signaling Technology. Anti-caveoline 2 was purchased from EPITOMICS.

2.2. Cell culture and application of reagents

Human neuroblastoma cells SH-SY5Y were obtained from the ATCC. Basal culture conditions for SH-SY5Y have been described previously [19]. Briefly, SH-SY5Y cells were maintained at 37 °C in 5% CO_2 in DMEM/F12 medium with or without phenol red, supplemented with 5% fetal bovine serum, 10,000 U/ml penicillin G, and 100 mg/ml streptomycin. Culture medium was exchanged twice a week during cell growth. Experiments were performed using 5×10^5 cells per well in 6-well culture plates for immunoblots, 1×10^3 cells per well in 8-well chamber slides for immunohistochemistry, and 3×10^3 cells per well in 96-well culture plates for viability assays. Cells were differentiated into neural lineages by incubation for 2–3 days with 5 μM RA. PD-related toxins (MPP^+, MG132, and thapsigargin) and T3s were added to the medium simultaneously and incubated for the duration indicated in the figure legends. Kinase inhibitors or receptor antagonists were added to the medium at 1 h or 30 min, respectively, before treatment with T3s. To transfect the Akt-PH vector (gifted by Dr. Ohno) [25] into SH-SY5Y cells, TransFast™ Transfection Reagents (Promega, Madison, WI, USA) were used in accordance with the manufacturer's protocol.

2.3. Cell viability assay (MTT assay)

Cell viability was measured using the MTT assay following the protocol described previously with some modifications [19]. Specifically, 3×10^3 cells/well were seeded in 96-well plates, cultured for 2 or 3 days with RA, and then toxins and T3s were added to the culture medium. After 48 h, cells were incubated with MTT for 2 h at 37 °C. After adding 100 μl of 0.04 N HCl in 2-propanol and mixing thoroughly to dissolve the dark blue crystals, the MTT reduction was measured with a microplate reader (Bio-Rad; wavelength of 570 nm). Data are presented as percent post-treatment recovery (percent live cells), where the absorbance from the control non-treated cells was defined as 100% live cells.

2.4. Immunobloting and immunofluorescent staining

For immunoblots, cultured cells were harvested from 6-well culture plates, and lysed in SDS sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, and 2 mM EDTA). Aliquots (20 μg) were separated on the basis of molecular size on 7.5–12% polyacrylamide gels, transferred onto polyvinylidene difluoride membranes (Hybond-P; GE Healthcare, Buckinghamshire, UK), and hybridized with the required antibody in PBS-Tween 20 at room temperature (RT) for 1 h. The immunoreactive signal was detected using horseradish peroxidase-linked anti-rabbit IgG and ECL detection reagents (GE Healthcare). Protein content of each sample was measured using the BCA protein assay system (Thermo Fisher Scientific Inc., Rockford, IL, USA). The density of immunoblot signal (expressed as the ratio of phosphorylated/total protein for Akt, Erk, p38 and JNK) was semi-quantified using Image J software.

For immunofluorescent staining, 4% paraformaldehyde-fixed SY-SY5Y cells were washed with PBS, and incubated with ice-cold solution containing 95% ethyl alcohol and 1% acetic acid for 5 min. After washing with PBS twice, cells were incubated with antibodies overnight at 4 °C. Then, cells were washed twice with PBS and incubated with a Texas Red-conjugated secondary antibody at RT for 4 h. Cells were washed with PBS several times and finally covered using Vectashield (Vector Lab Inc., Burlingame). Nuclei were stained using Hoechst 33342. Immunohistochemical signals were detected using fluorescent microscopy.

2.5. Binding analysis between T3s and ER

To analyze binding between T3s and ERα or ERβ, competitive radiometric binding assays [26] were carried out. Full-length ERα or ERβ protein (0,5 nM) and ^{3}H-E2 (1 nM) were incubated in 500 μl of binding buffer (10 mM Tris–HCl, pH 7.4, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol, and 1% bovine serum albumin) with 10^{-9}–10^{-4} M T3s or 10^{-12}–10^{-6} M E2 at 30 °C for 1 h. After incubation, the reaction was terminated by adding 200 μl of activated charcoal suspension. After 1 h of absorption, ER-bound ^{3}H-E2 and unbound ^{3}H-E2 (absorbed by the charcoal) were separated by centrifugation for 3 min at 1000 ×*g*.

Aliquots (500 μl) of the supernatant containing ERα/ERβ were transferred to another tube, and 3 ml of scintillation cocktail (Perkin Elmer)

was added. The radiometric counts from ^{3}H-E2 were measured with a liquid scintillation counter.

2.6. Gene silencing

Gene silencing of ERα, ERβ, caveolin-1, caveolin-2, and caveolin-3 was performed by RNA interference. The siRNA sequences for ERα and ERβ were designed in accordance with a previous report [27] and purchased from Sigma Genosys. Control siRNA, caveolin-1 siRNA, caveolin-2 siRNA, and caveolin-3 siRNA were purchased from Santa Cruz (Santa Cruz, CA, USA). Transfection of siRNAs was performed using the siRNA Reagent System (Santa Cruz) in accordance with the manufacturer's protocol.

2.7. Antibody array

Cell lysates were prepared as recommended by Kinexus Bioinformatics Corporation (Vancouver, BC, Canada). Protein extracts from SH-SY5Y cells treated with γT3 (1 μM, 2 h) or the vehicle were collected, and their protein profiles were compared using the Kinexus™ antibody array system following the manufacturer's protocol.

2.8. Statistical analyses

Quantitative data were assessed by ANOVA using the Stat View software. The criterion for statistical significance was $p < 0.01$ for the cell viability assay, and $p < 0.05$ for semi-quantitative data obtained from immunoblotting. All values are expressed as means ± S.E.

3. Results

3.1. T3s exhibit a cytoprotective effect against PD-related toxicities

In order to clarify the cytoprotective effect of T3s against PD-related toxicities, we established cell toxicity models of PD using the human dopaminergic neuroblastoma cell line, SH-SY5Y. Although the mitochondrial toxin MPP^+ (2 mM) reduced cell viability to around 40% at 48 h after treatment, simultaneous treatment with T3s significantly increased viability in a dose-dependent manner (Fig. 1A–D). The protective effects of γT3 (Fig. 1C) and δT3 (Fig. 1D) were more remarkable than those of αT3 and βT3 (Fig. 1A, B). We also investigated the effect of simultaneous T3 treatment (1 μM) against cell death induced by the proteasome inhibitor MG132 (5 μM) (Fig. 1E) or the endoplasmic reticulum stressor thapsigargin (1 μM) (Fig. 1F). Treatment with αT3, βT3, γT3, or δT3 reduced the amount of cell death induced by a 48-h exposure to MG132 (Fig. 1E). The cytoprotective effects of γT3 and δT3 were superior to those of αT3 and βT3, as well as the protective effect against MPP^+ (Fig. 1A–D). Similarly, the cytoprotective effects of γT3 and δT3 against thapsigargin were significant; however, those of αT3 and βT3 were mild and were not statistically significant (Fig. 1E).

To confirm whether these cytoprotective effects were dependent on the antioxidative action of T3s, we measured lipid hydroperoxide and protein carbonyl as indices of oxidative stress. Although each T3 showed a tendency to reduce the production of lipid hydroperoxide induced by MPP^+ exposure, this antioxidative effect was not statistically significant (Supplementary Fig. 1A). Statistical differences in the level of protein carbonyl when compared with and without T3 treatment were not observed with regard to MPP^+-induced toxicity (Supplementary Fig. 1B). Since the cytoprotective effects of γT3 and δT3 were superior to those of the other T3s, the subsequent experiments were performed using mainly these two compounds.

3.2. γT3 and δT3 activate PI3K/Akt signaling in SH-SY5Y cells

Although vitamin E is an established antioxidative agent, the T3s were cytoprotective against agents with oxidative stress-independent toxicities, including a proteasome inhibitor and an activator of endoplasmic reticulum stress, as shown in Fig. 1. Therefore, we hypothesized that the cytoprotective effect of T3s is, at least in part, also dependent on intracellular signal transduction. In order to elucidate which signaling pathways might be involved, we investigated the activation of the MAPK and PI3K/Akt pathways as candidate signals for the cytoprotective action of T3s. As seen in Fig. 2, Akt was phosphorylated at 120 min after T3 treatment, particularly in response to γT3 and δT3 (Fig. 2A–D). We also assessed the phosphorylation of Erk1/2 (Fig. 2E–H), p38 (Fig. 2I–L), and JNK (Fig. 2M–P). Erk1/2 was significantly phosphorylated upon γT3 and δT3 treatments, but not by αT3 and βT3 (Fig. 2E–H). Two other MAPKs, p38 and JNK, were not significantly phosphorylated by treatment with T3s under our experimental conditions (Fig. 2I–P).

We examined the translocation of Akt using PH-Akt–GFP-expressing cells because translocation of Akt to the proximity of the plasma membrane from the cytosolic area is a robust index of Akt activation. Treatment of γT3 and δT3 induced translocation of PH-Akt–GFP from the cytoplasm to the plasma membrane area (Fig. 2Q–S), suggesting that γT3 and δT3 can activate the Akt/PI3K signaling pathway.

Based on these results, we examined whether a panel of kinase inhibitors could inhibit the cytoprotective effects of γT3 and δT3. Neither PD98059 (5 μM) nor SB203580 (5 μM) (inhibitors of Erk1/2 and p38, respectively) reversed the cytoprotective effects of γT3 (Fig. 2T) and δT3 (Fig. 2U). However, strikingly, the PI3K inhibitors LY29004 (10 μM) and wortmannin (2 μM) dramatically blocked γT3-dependent protection. The JNK inhibitor SP600125 (5 μM) also showed an inhibitory effect (Fig. 2T, U), which we attribute to suppression of the mechanism by which MPP^+ itself triggers toxicity.

3.3. Estrogen receptor β plays an important role in the cytoprotective effects of γT3 and δT3

In neuronal cells, several receptors, including Trk neurotrophin receptors, dopamine receptors, adenosine receptors, and ERs, have been reported as upstream mediators of PI3K/Akt signaling. Therefore, we investigated the association of several receptors with T3-related cytoprotection using chemical inhibitors. The dopamine D2 receptor antagonist droperidol (10 μM), the neurotrophin receptor (Trk) inhibitor K252a (3 nM), and the adenosine A_{2A} receptor antagonist 8-(chlorostyryl) caffeine (10 μM) did not inhibit the cytoprotective effects of γT3 and δT3 against MPP^+ toxicity (Fig. 3A, B). On the other hand, the ER antagonist tamoxifen (1 μM) inhibited the cytoprotective effects of both γT3 and δT3 (Fig. 3A, B).

The tamoxifen-dependent inhibition of γT3 and δT3 activities indicates that ER signaling is associated with their cytoprotective effects. Since docking simulation has previously shown that δT3 can bind to ERβ, we measured binding activity between T3s and ERα/ERβ using a radiometric competitive inhibition method [26] (Fig. 4). This showed direct binding of γT3 and δT3 to ERβ, with the latter interaction being more pronounced. On the other hand, the binding activities of αT3 and βT3 to ERβ or of αT3, βT3, γT3, δT3 to ERα were weak (Fig. 4). Although all T3s have a common farnesyl isoprenoid tail, the structure of the chromanol ring differs between individual members. We thus infer that the different ERβ-binding activities exhibited by T3s may depend on the number and location of methyl groups on the chromanol ring.

We next investigated the translocation of ERβ under γT3 and δT3 treatments. In SH-SY5Y cells, ERβ localized mainly in extranuclear spaces such as the cytoplasm and plasma membrane. Treatment with γT3 or δT3 induced the translocation of ERβ from cytosolic and/or membrane areas to the perinuclear space (Supplementary Fig. 2). These results suggest that γT3 or δT3 also binds to ERβ in SH-SY5Y cells and that ERβ-related signaling may play an important role in cytoprotection.

To clarify whether the cytoprotective effect of γT3 and δT3 is due to ERβ specifically, rather than ERα, we performed gene silencing of ERα and ERβ by RNA interference (Fig. 5A). Knockdown of ERβ prevented

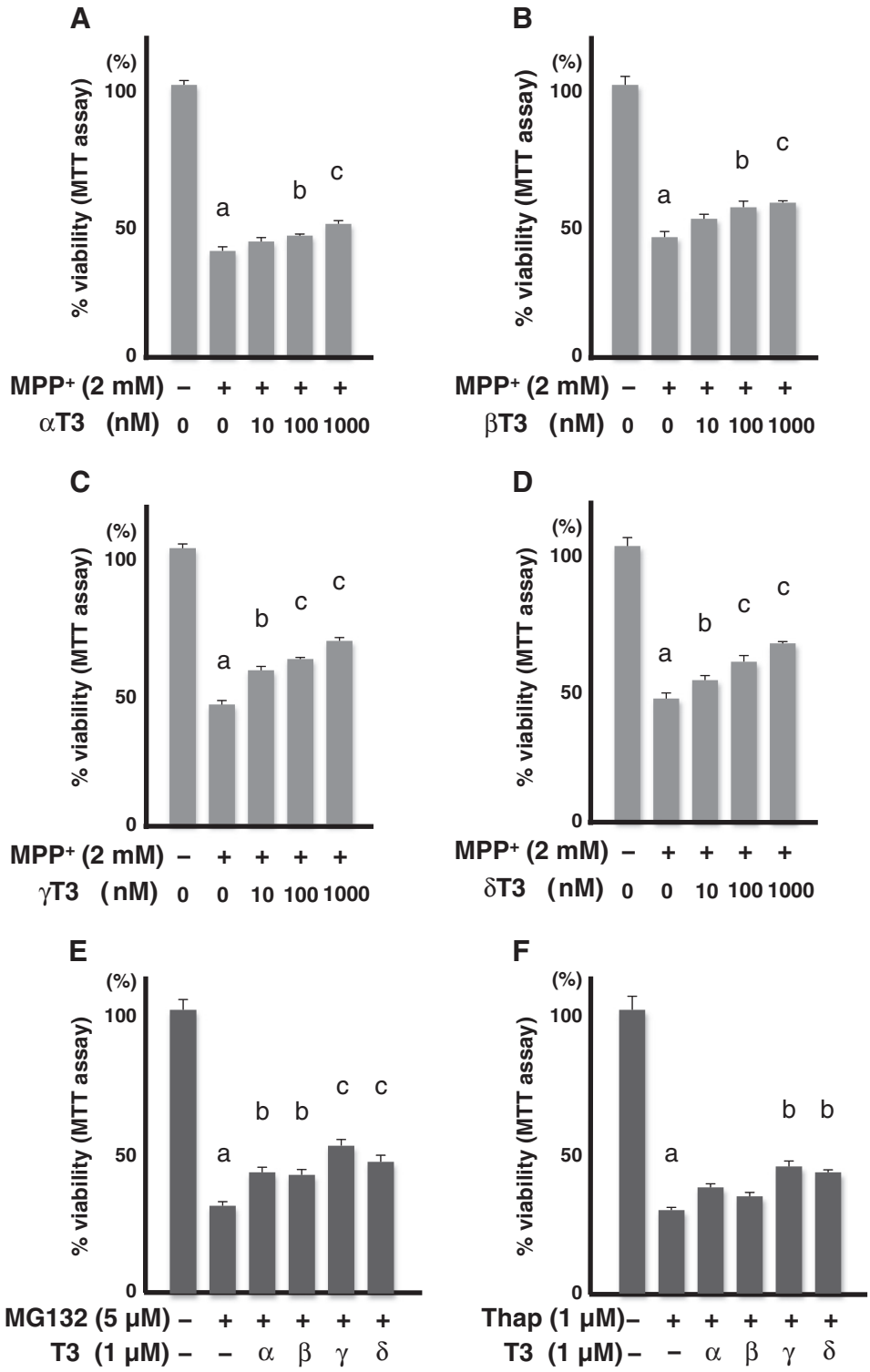

Fig. 1. T3s exhibit a cytoprotective effect against PD-related toxicities in SH-SY5Y cells. (A–D) T3s are cytoprotective against MPP^+ (2 mM) in a dose-dependent manner. T3s and MPP^+ were added simultaneously, and viabilities were measured at 48 h after treatment using the MTT assay. a: $p < 0.001$ vs $MPP^+(-)$/T3s(−), b: $p < 0.01$ vs MPP^+ only, c: $p < 0.001$ vs MPP^+ only (ANOVA). (A, B) αT3 and βT3 are mildly protective against MPP^+. (C) γT3 and δT3 are strongly protective against MPP^+. (E) T3s are cytoprotective against the proteasome inhibitor MG132 (5 μM). T3s (1 μM) and MG132 were added simultaneously, and viabilities were measured at 48 h after treatment, by using the MTT assay. a: $p < 0.001$ vs MG132(−)/T3s(−), b: $p < 0.01$ vs MG132 only, c: $p < 0.001$ vs MG132 only (ANOVA). (F) T3s are cytoprotective against the endoplasmic reticulum stressor thapsigargin (Thap) (1 μM). T3s (1 μM) and Thap were added simultaneously, and viability was measured at 48 h after treatment using the MTT assay. a: $p < 0.001$ vs Thap (−)/T3s(−), b: $p < 0.01$ vs Thap only (ANOVA).

the cytoprotective effect of γT3 or δT3 (Fig. 5B, C), whereas the knockdown of ERα showed no inhibitory effect against γT3 and had only mild effects against δT3 (Fig. 5B, C). The partial effects of ERα knockdown may be attributable to the fact that ERα homodimerizes or heterodimerizes with ERβ on the cell membrane. We also investigated the inhibitory effect of ERβ knockdown on the phosphorylation of Akt. ERβ knockdown significantly inhibited the γT3 or δT3-induced phosphorylation of Akt (Fig. 5D, E).

ER signaling includes genomic (i.e., modulation of gene expression) and non-genomic pathways. We first examined the ER-dependent

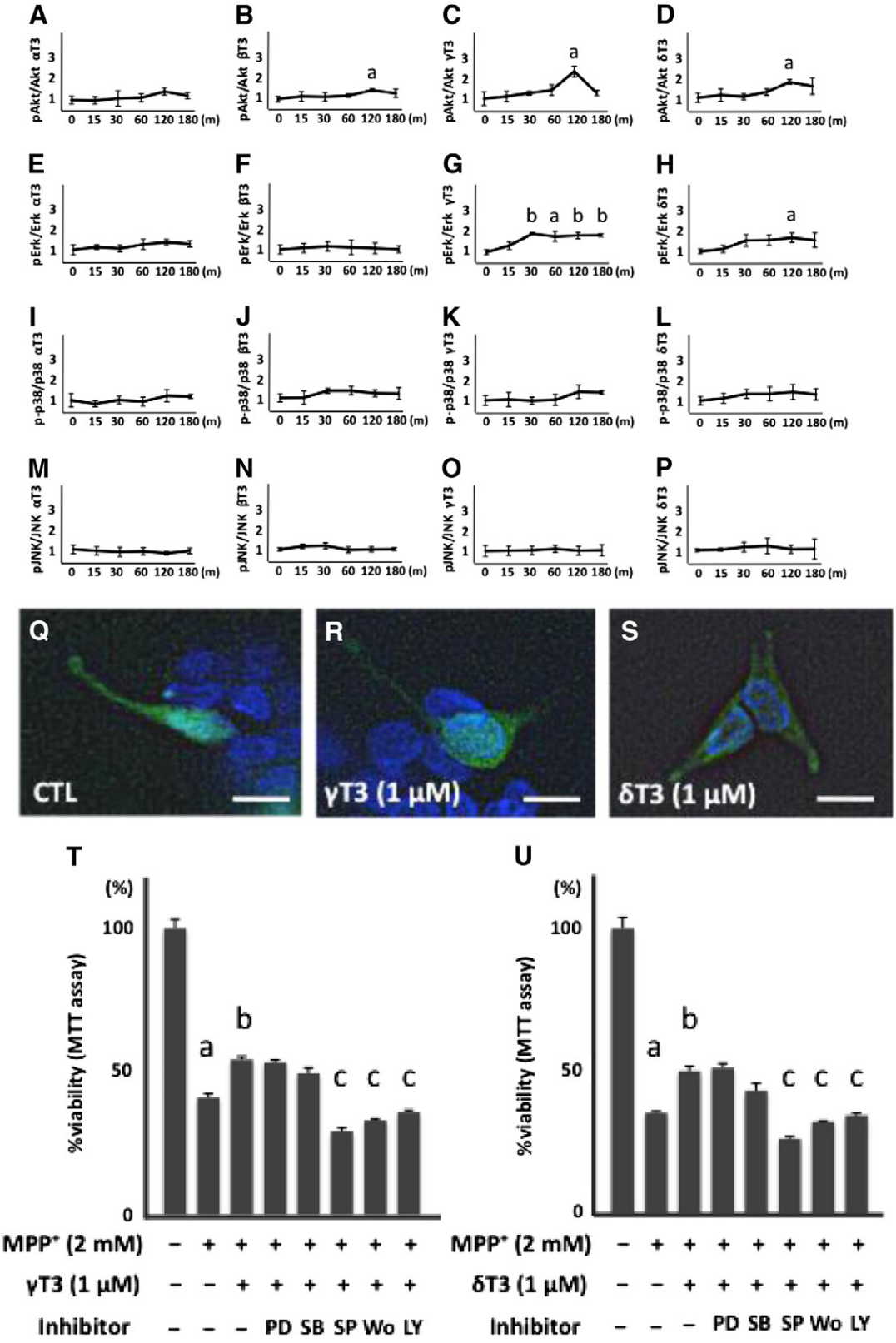

Fig. 2. T3s, especially γT3 and δT3, activate the PI3K/Akt pathway. Semi-quantitative data of the pAkt/Akt blots (A–D), pErk/Erk (E–H), p-p38/p38 (I–L), and pJNK/JNK (M–P). Cell extracts were collected several minutes after αT3 (1 μM) (A, E, I, M), βT3 (1 μM) (B, F, J, N), γT3 (1 μM) (C, G, K, O), or δT3 (1 μM) (D, H, L, P) treatment. β, g (D, H, L) treatment leads to Akt phosphorylation after 120 min. a: $p < 0.05$ vs 0 min (ANOVA) (B, C, D). Erk is phosphorylated by gT3 or dT3 (G, H). a: $p < 0.05$ vs 0 min, b: $p < 0.01$ vs 0 min (ANOVA). The GFP-fused PH domain of Akt was translocated from the cytoplasm to the plasma membrane (Q–S). Scale bar = 10 μm. (Q) PH-Akt–GFP was located in the cytosolic area in untreated cells (CTL), (R) PH-Akt–GFP was translocated to the plasma membrane at 90 min after treatment with γT3 (1 μM), and (S) PH-Akt–GFP was translocated to the plasma membrane at 90 min after treatment with δT3 (1 μM).(T, U) Kinase-inhibitor experiments. PD: Erk inhibitor PD98059, SB: p38 inhibitor SB203580 (10 μM), SP: JNK inhibitor SP600125 (10 μM), Wo and LY: PI3K inhibitor wortmannin (2 μM) and LY294002 (10 μM), respectively. Each inhibitor was added to the culture medium at 30 min before MPP^+ (2 mM) and T3s (1 μM). a: $p < 0.001$ vs $MPP^+(-)$/T3s(−), b: $p < 0.01$ vs MPP^+ only, c: $p < 0.001$ vs MPP^+/T3s (ANOVA). (T) The PI3K inhibitors Wo and LY prevented the cytoprotective effect of γT3 against MPP^+ toxicity. The JNK inhibitor SP also prevented the cytoprotective effect of γT3. (U) The PI3K inhibitors Wo and LY prevented the cytoprotective effect of δT3 against MPP^+ toxicity, as did the JNK inhibitor SP.

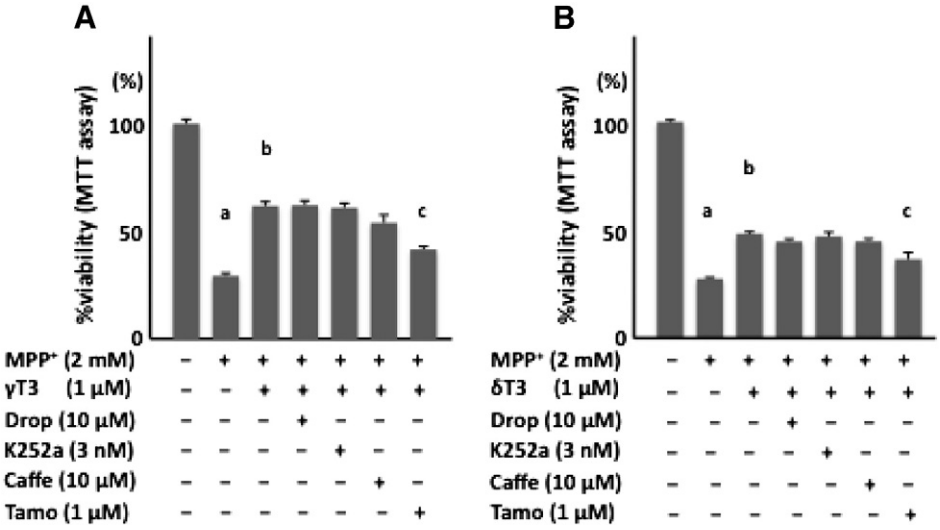

Fig. 3. Tamoxifen, an ER inhibitor, prevents the cytoprotective effect of γT3 and δT3. The ER inhibitor tamoxifen (Tamo, 1 μM), but not the dopamine D2 receptor antagonist droperidol (Drop), Trk neurotrophin receptor antagonist K252a (3 nM), or the adenosine A_{2A} receptor antagonist 8-(chlorostyryl) caffeine (Caff, 10 μM), abrogated the cytoprotective effect of γT3 (A) and δT3 (B) in SH-SY5Y cells treated with MPP^+. Viability was measured at 48 h after MPP^+ and T3 treatment. a: $p < 0.001$ vs $MPP^+(-)$/T3s(−), b: $p < 0.001$ vs MPP^+ only, c: $p < 0.001$ vs MPP^+/T3s (ANOVA).

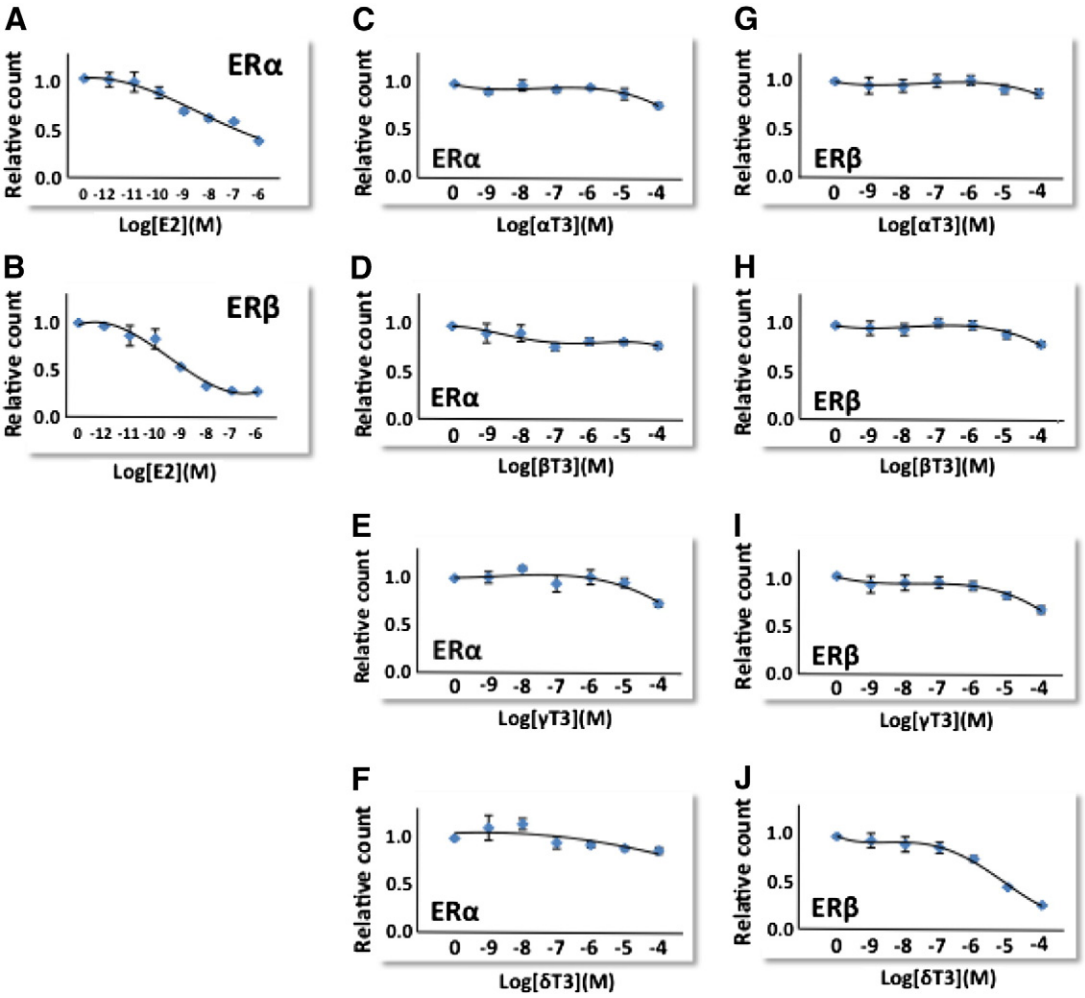

Fig. 4. Competitive radiometric binding assay for T3s to ERα and ERβ. (A) Competitive radiometric binding of free E2 to ERα. (B) Competitive radiometric binding of free E2 to ERβ. (C–F) Competitive radiometric binding of T3s to ERα. Data show weak binding between T3s and ERα. (G–J) Competitive radiometric binding of T3s to ERβ. γT3 (I) and δT3 (J) competitively inhibit binding between ERβ and ^{3}H-E2 in a ligand concentration-dependent manner, suggesting that both γT3 (I) and δT3 (J) can bind to ERβ.

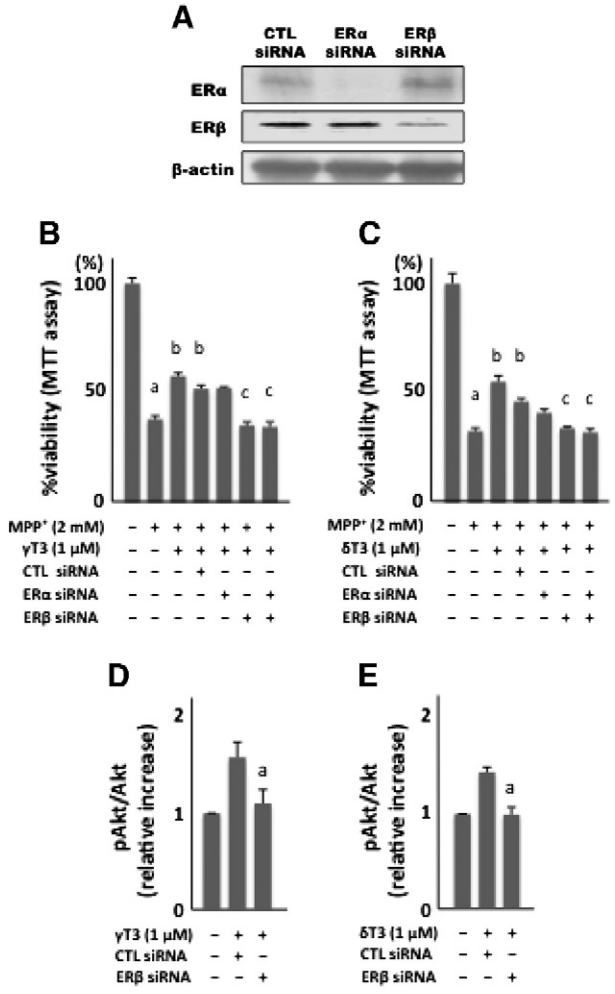

Fig. 5. ERβ is a key molecule in the γT3 or δT3-related cytoprotection mechanism against MPP^+ in SH-SY5Y cells. (A) Gene silencing of ERα and ERβ using siRNA. (B) Gene silencing of ERβ abrogated the cytoprotective effect of γT3 in cells treated with MPP^+. a: $p < 0.001$ vs $MPP^+(-)/\gamma T3(-)$, b: $p < 0.001$ vs MPP^+ only, c: $p < 0.001$ vs $MPP^+/\gamma T3$/CTL siRNA. (C) Gene silencing of ERβ abrogated the cytoprotective effect of δT3 in cells treated with MPP^+. a: $p < 0.001$ vs $MPP^+(-)/\delta T3(-)$, b: $p < 0.001$ vs MPP^+ only, c: $p < 0.001$ vs $MPP^+/\delta T3$/CTL siRNA. (D) Gene silencing of ERβ prevented the phosphorylation of Akt following γT3 treatment. a: $p < 0.05$ vs no treatment, b: $p < 0.05$ vs γT3 treatment. (E) Gene silencing of ERβ prevented the phosphorylation of Akt upon δT3 treatment. a: $p < 0.05$ vs no treatment, b: $p < 0.05$ vs δT3 treatment.

genomic signaling by evaluating the levels of the downstream transcriptional targets, cathepsin D and Egr-1. In HeLa cells, E2, γT3, and δT3 upregulated both transcripts; however this was not observed in SH-SY5Y cells (Supplementary Fig. 3). These results suggest that, at least in SH-SY5Y cells, ERβ may signal via the non-genomic pathway upon activation by T3s.

It has been reported that phenol red can bind to ERs, and in doing so exhibit estrogenic effects [28]. However, the protective effect of γT3 and δT3 was similar in phenol red-free medium and regular medium (Supplementary Fig. 4). Therefore, regular medium was used in all experiments.

3.4. Caveola formation is important for the cytoprotective effect of γT3 and δT3

In order to characterize the immediate signaling events triggered by T3 treatment, we utilized a Kinexus™ antibody array. Molecules that had a high z-ratio (z-ratio > 1.2) and were upregulated by γT3 treatment (1 μM, 2 h) in SH-SY5Y cells are listed in Table 1. Interestingly, 4 of the top 9 molecules were classified as endocytosis-related or actin/microtubule modifying-related molecules. This finding suggests that γT3/ERβ signaling may involve endocytotic action, such as caveola formation, as an early step.

Table 1
The list of upregulated protein (z-ratio > 1.2) from antibody microarray screening (Kinexus™ antibody array).

Molecule	Antibody	Character	
ATF2	Pan-specific	Activating transcription factor 2 (CRE-BP1)	
Cofilin 1	Pan-specific	Actin modulating protein, endocytosis	[a]
Caveolin 2	Pan-specific	Caveolae-related protein	[a]
Aurora A	Pan-specific	Aurora kinase, stabilization of microtubulas, regulator of mitotic call division	[a]
CREB1	S133	cAMP response element binding protein	
CDK5	Pan-specific	Cyclin-dependent protein kinase, regulator of endocytosis	[a]
COT	Pan-specific	Osaka thyroid oncogene protein-serine kinase (Tpl2)	
eIF4E	S20S	Eukaryotic translation initiation factor 4	
Erkl/2	T202 + Y204	Extracellular regulated protein-serine kinase 1 + 2	

[a] Endocytosis-related and/or actin/microtubules-related protein.

Receptors on the plasma membrane are usually localized in lipid rafts. The binding of extracellular ligands to receptors triggers the formation of caveolae, which are vesicular structures involving scaffolded membrane domains. Transport in caveolae ensures that extracellular signals are transported to intracellular organelles for degradation or recycling to the surface. It has been reported that the ER is found within caveolae at the plasma membrane, and caveolae are believed to link estrogen uptake to the non-genomic activities of ERs [29]. Therefore, we hypothesized that T3/ERβ-mediated signaling may involve caveola formation. We investigated whether knockdown of caveolin, an integral component of caveolae, inhibited the cytoprotective effects and Akt phosphorylation in response to T3 treatment. Knockdown of caveolin-1 (Fig. 6A) and caveolin-2 (Fig. 6B), prevented the cytoprotective effects of γT3 and δT3 (Fig. 6C, D). Interestingly, caveolin knockdown did not inhibit the Akt phosphorylation induced by γT3 and δT3 treatments, suggesting that the activation of PI3K/Akt may be an upstream mediator of caveola formation. Knockdown of caveolin-3, which is generally a muscle-specific isoform, also prevented the cytoprotective effect of T3s, and did not inhibit Akt phosphorylation (Supplementary Fig. 5).

4. Discussion

The protective effect of T3s in neurodegenerative disease models has received considerable attention [4,24]. Since vitamin E is well known as an antioxidant, many previous studies have suggested that the protective effect of T3s depends upon their antioxidative effects in neuroblastoma cell lines [30,31]. However, there is evidence that other cytoprotective mechanisms of T3s involving intracellular signal cascades may be at play in neurodegenerative disease models [24]. Therefore in this study, we focused upon antioxidant-independent cytoprotective effects of T3 and investigated the detailed mechanisms of T3-induced cytoprotection using a cellular model of PD. It has been reported that SH-SY5Y cells differentiated by 10 μM RA plus 1% FBS for 7 days is suitable for studying PD [32]. However, we used SH-SY5Y cells differentiated by 5 μM RA and 5% FBS for a short period (2–3 days), because an effect of RA-associated PI3K activation should be reduced.

Recently, neuroprotection has been proposed as a therapeutic strategy for the treatment of PD [17–19]. Oxidative stress contributes, at least in part, to the pathogenic cascade leading to dopaminergic neuronal degeneration in PD. Since αTOC, the most common derivative of vitamin E, is well known as an antioxidant, it represents a promising candidate as a therapeutic agent for PD [8,9]. Moreover, some epidemiologic studies suggest that sufficient intake of vitamin E is protective against the onset of PD. However, antioxidative therapy for PD using exogenous antioxidants, including αTOC, has not been successful thus far

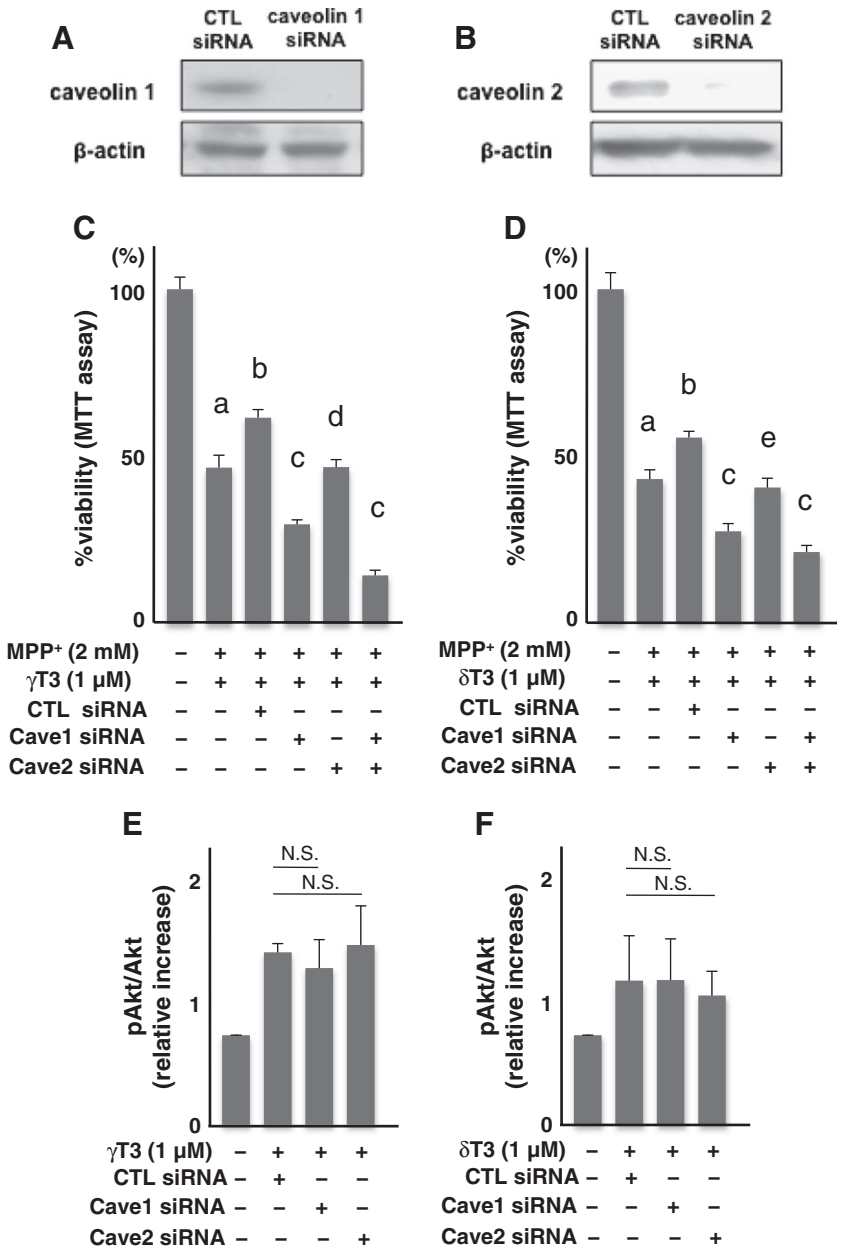

Fig. 6. Caveola formation, including that of caveolin protein, is important in the cytoprotective effects of γT3 and δT3. (A) Gene silencing of caveolin-1 via specific siRNA. (B) Gene silencing of caveolin-2 via specific siRNA. (C) Gene silencing of caveolin-1 (cave1) and 2 (cave2) prevented the cytoprotective effect of γT3 against MPP^+ toxicity. a: $p < 0.001$ vs $MPP^+(-)/\gamma T3(-)$, b: $p < 0.001$ vs MPP^+ only, c: $p < 0.001$ vs $MPP^+/\gamma T3$/CTL siRNA and $MPP^+/\gamma T3$/cave2 siRNA, d: $p < 0.001$ vs $MPP^+/\gamma T3$/CTL siRNA. (D) Gene silencing of cave1 and cave2 abrogated the ability of δT3 to protect against MPP^+ toxicity. a: $p < 0.001$ vs $MPP^+(-)/\delta T3(-)$, b: $p < 0.001$ vs MPP^+ only, c: $p < 0.001$ vs $MPP^+/\delta T3$/CTL siRNA and $MPP^+/\delta T3$/cave2 siRNA, e: $p < 0.01$ vs $MPP^+/\delta T3$/CTL siRNA. (E, F) Gene silencing of cave1 and cave2 did not prevent the phosphorylation of Akt upon γT3 (E) or δT3 (F) treatment. N.S., not significant.

[14]. Therefore, we focused upon the non-antioxidative effects of the other vitamin E family members, namely the T3s.

In this study, γT3 and δT3 were particularly effective in their protection against PD-related toxicities. T3s did have mild antioxidative effects, as shown in Supplementary Fig. 1. However, it is difficult to conclude whether this contributes to their cytoprotective activity for the following reasons: (i) the antioxidative effect of T3 was not statistically significant in the MPP^+ treatment model (Supplementary Fig. 1A, B) and (ii) T3s also protected against the proteasome inhibitor MG132 and the endoplasmic reticulum stressor thapsigargin, neither of which are directly associated with induction of oxidative stress (Fig. 1B, C). These observations suggest that T3s, and in particular γT3 and δT3, exert both antioxidant-dependent and -independent effects.

Since the PI3K/Akt and the MAPK pathways play an important role in neuroprotection [19,23,24,33], we investigated these signal transduction systems as candidates in our model. Akt was significantly phosphorylated at 120 min after γT3 and δT3 treatments, and PI3K inhibition dramatically reduced the cytoprotective effects of γT3 and δT3 (Fig. 2), suggesting that activation of PI3K/Akt is a key signal for the cytoprotective effects of γT3 and δT3. Erk was also mildly phosphorylated upon γT3 or δT3 treatment, but this effect was less robust than that observed for Akt; additionally, the Erk inhibitor did not reduce T3-dependent cytoprotection (Fig. 2). The JNK inhibitor SP600125 also antagonized γT3/δT3-mediated cytoprotection (Fig. 2). We consider that this was related more to suppression of the mechanism by which MPP^+ is usually converted to a toxic molecule, rather than to blockade of T3-dependent signaling per se.

We also attempted to identify factors acting upstream of PI3K/Akt signaling. From previous reports, several receptor-related signals have been identified, such as the Trk neurotrophin receptor [19,34,35],

adenosine A_{2A} receptor [36], dopamine D2 receptor [37], ERs [38], and insulin receptor [39]. In this study, we targeted the Trk receptor, A_{2A} receptor, D2 receptor, and ERs as potential factors upstream of PI3K/Akt since they are reported to be important for neuroprotection. Interestingly, the ER antagonist tamoxifen inhibited the cytoprotective effects of γT3 and δT3 (Fig. 3), suggesting that the ER may be an initial target for these T3s. Therefore, we tried to identify which isoform of ER, ERα or ERβ, was more critical for the effect of these tocotrienols. An ER/T3 binding assay showed that the most prominent interactions were between γT3 and δT3 and ERβ However, this binding was much weaker when compared to that between ERβ and its natural ligand, E2, as shown by the high concentrations of T3s required to detect interaction. Recently, using a software-based approach, Comitato et al., reported that δT3 can bind ERβ and that the 5-hydroxyl group on the chromanol ring of δT3 is important for direct binding [26]. Our T3s are highly purified when compared to those described in previous reports, and by comparing several types of T3, we found that δT3 and γT3 can bind to ERβ (Fig. 4). Considering the structures of T3, we suggest that that the differences in binding activity of the T3 family members to ERβ are due to variations in the number and location of methyl groups on the chromanol ring. We hypothesize that T3 members with lower number of methyl groups located on the chromanol ring and around the 5-hydroxyl group are more likely to bind tightly to ERβ.

Moreover, we investigated the functional interaction between γT3/δT3 and ERβ using siRNA. Knockdown of ERβ clearly prevented the cytoprotective effects of γT3 and δT3, whereas ERα knockdown modestly inhibited the cytoprotective effect of δT3 (Fig. 5C). We hypothesize that the inhibitory effect of ERα knockdown is indirectly due to the effect of ERβ, since ERα and ERβ can heterodimerize with each other on the plasma membrane [40].

Interestingly, although the binding between ERβ and δT3 showed higher affinity than that between ERβ and γT3, the cytoprotective effects of both compounds were almost identical. This discrepancy may be due to slight differences in their mechanisms of action. For example, γT3 was a more robust activator of Akt and was also a more potent reducer of lipid hydroperoxide when compared with other T3s. The cytoprotective mechanisms of γT3 may extend beyond its ERβ-related activity.

Upon stimulation of ERα and ERβ by estrogen, the receptors translocate to the nucleus and act as transcription factors that modify the activity of target genes [38,41]. In addition to this conventional activity, estrogen also utilizes plasma membrane ERs, and thus the two pathways are often referred to as “classic genomic” and “non-genomic” pathways [38]. The classical genomic activity of ERs generally requires prolonged estrogen exposure [42]. On the other hand, the non-genomic action of estrogen on membrane-associated ERs is rapid and leads to the activation of membrane-associated signaling molecules, such as PI3K and MAPKs [38,43]. Membrane localization of ERs in SH-SY5Y cells [44] and several other cell types [45,46] has been reported previously. Furthermore, it has been reported that S-palmitoylation is important for ER localization at the plasma membrane [47]. In order to confirm which pathway was predominant in the cytoprotective role of T3s in SH-SY5Y cells, we examined the induction of the genetic pathway-related molecules, cathepsin D and Egr-1, under γT3 or δT3-treated conditions. Since neither molecule was induced by T3s in SH-SY5Y cells, we suggest that the protective effect of T3 in SH-SY5Y cells may depend on non-genomic, rather than genomic, pathways.

In order to characterize the events proximal to T3 treatment, we carried out differential screenings for proteins using a Kinexus™ antibody array. This analysis showed that, of the top 9 molecules, 4 were classified as endocytosis-related (e.g., cofilin 1, caveolin-2, CDK5) or as modifiers of actins or microtubules (e.g. cofilin 1, Aurora A). Therefore, we hypothesized that the T3/ER signal may involve endocytotic action, such as caveola formation, as an early step. Indeed, recent reports suggest that the non-genomic actions of ER are linked to caveola formation [29]. Caveolae are one of the factors that can induce receptor-mediated endocytosis [46,48]. Caveolae are present in the plasma membrane of most cell types and are thought to form membrane macrodomains or lipid rafts. The major structural protein of caveolae are caveolins [46], which are a family of unusual integral membrane proteins that each insert a hydrophobic loop into the membrane from the cytosolic side but do not extend across the plasma membrane. In order to confirm that caveolae are associated with T3/ER signaling we silenced caveolin-1, caveolin-2 (Fig. 6), and caveolin-3 (Supplementary Fig. 5). Under our experimental conditions, caveolin knockdown dramatically reduced the cytoprotective effects of γT3 and δT3. However, phosphorylation of Akt was not prevented by caveolin knockdown, suggesting that γT3/δT3 activation of Akt may occur prior to caveola formation.

Oxidative stress contributes to the pathogenic cascade leading to dopaminergic neuronal death in PD [49]. Furthermore, mitochondrial disturbance, which induces the generation of reactive oxygen species and causes secondary oxidative stress, is also important in the pathogenesis of PD [16,50]. However, antioxidative therapy against PD has not proved successful thus far. Because most antioxidants have very short half-lives, it has been difficult to achieve a prolonged effect during the long-term progression of neurodegenerative diseases such as PD. Neuroprotective drugs and food ingredients that are not associated with antioxidant properties should also be considered as treatments to delay the onset or progression of PD. Although T3s have an antioxidative effect, other cytoprotective mechanisms of T3s should be considered. In this study, we have therefore clarified the cytoprotective effects of T3s, and in particular those of γT3 and δT3. In summary (Fig. 7), γT3/δT3 binds to ERβ, which then forms a homodimer of ERβ or a heterodimer with ERα, on the plasma membrane, leading to activation of PI3K/Akt signaling. These events may trigger caveola formation via receptor-mediated endocytosis. After this early event, a non-genomic pathway causes ER upregulation (Fig. 7). Since ERβ is expressed not only in SH-SY5Y cells but also in the dopaminergic neurons of the substantia nigra of the mouse brain (our unpublished observations), these intracellular signals may also be activated by T3 administration in the PD brain.

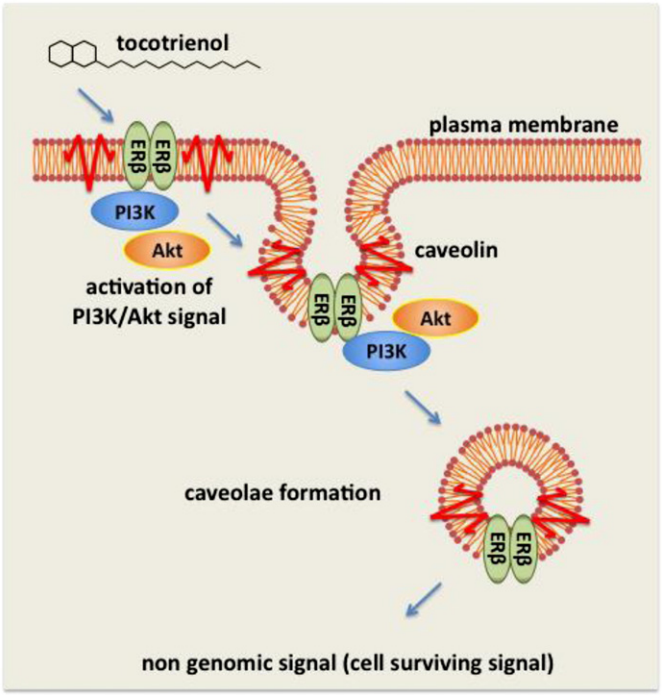

Fig. 7. Schematic diagram of the T3-dependent cytoprotective signaling pathway. T3s bind to ERb directly, and this ERβ signal then induces activation of the PI3K/Akt pathway, which triggers caveola formation involving ERβ. These initial events constitute an estrogen-dependent non-genomic pathway that is cytoprotective.

Our data have demonstrated a novel cytoprotective mechanism that is employed by the tocotrienols γT3 and δT3 and includes membrane ERβ/PI3K/Akt signaling via caveola formation, and this effect is not dependent on the antioxidative effect of T3s. This finding suggests that the ERβ/PI3K/Akt pathway may be a promising candidate for novel PD-targeted therapeutic agents.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbadis.2014.04.008.

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References

[1] A. Kamal-Eldin, L.A. Appelqvist, The chemistry and antioxidant properties of tocopherols and tocotrienols, Lipids 31 (1996) 671–701.
[2] C.K. Sen, S. Khanna, S. Roy, Tocotrienols: vitamin E beyond tocopherols, Life Sci. 27 (2006) 2088–2098.
[3] C.E. Elson, Tropical oils: nutritional and scientific issues, Crit. Rev. Food Sci. Nutr. 31 (1992) 79–102.
[4] C.K. Sen, S. Khanna, S. Roy, Tocotrienols in health and disease: the other half of the natural vitamin E family, Mol. Aspects Med. 28 (2007) 692–728.
[5] L. Packer, Interactions among antioxidants in health and disease: vitamin E and its redox cycle, Proc. Soc. Exp. Biol. Med. 200 (1992) 271–276.
[6] E. Niki, Y. Yamamoto, M. Takahashi, E. Komuro, Y. Miyama, Inhibition of oxidation of biomembranes by tocopherol, Ann. N. Y. Acad. Sci. 570 (1989) 23–31.
[7] J. Atkinson, R.F. Epand, R.M. Epand, Tocopherols and tocotrienols in membranes: a critical review, Free Radic. Biol. Med. 44 (2008) 739–764.
[8] M.C. de Rijk, M.M. Breteler, J.H. den Breeijen, L.J. Launer, D.E. Grobbee, F.G. van der Meche, A. Hofman, Dietary antioxidants and Parkinson disease. The Rotterdam study, Arch. Neurol. 54 (1997) 762–765.
[9] S.M. Zhang, M.A. Hernan, H. Chen, D. Spiegelman, W.C. Willett, A. Ascherio, Intakes of vitamins E and C, carotenoids, vitamin supplements, and PD risk, Neurology 59 (2002) 1161–1169.
[10] M.W. Fariss, J.G. Zhang, Vitamin E therapy in Parkinson's disease, Toxicology 189 (2003) 129–146.
[11] M. Sano, C. Ernesto, R.G. Thomas, M.R. Klauber, K. Schafer, M. Grundman, P. Woodbury, J. Growdon, C.W. Cotman, E. Pfeiffer, L.S. Schneider, L.J. Thal, A controlled trial of selegiline, α-tocopherol, or both as treatment for Alzheimer's disease. The Alzheimer's disease cooperative study, N. Engl. J. Med. 336 (1997) 1216–1222.
[12] A. Ascherio, M.G. Weisskopf, E.J. O'Reilly, E.J. Jacobs, M.L. McCullough, E.E. Calle, M. Cudkowicz, M.J. Thun, Vitamin E intake and risk of amyotrophic lateral sclerosis, Ann. Neurol. 57 (2005) 104–110.
[13] G. Logroscino, K. Marder, L. Cote, M.X. Tang, S. Shea, R. Mayeux, Dietary lipids and antioxidants in Parkinson's disease: a population-based, case-control study, Ann. Neurol. 39 (1996) 89–94.
[14] Parkinson study group, Impact of deprenyl and tocopherol treatment on Parkinson's disease in DATATOP patients requiring levodopa, Ann. Neurol., 39, Parkinson study group, 1996. 37–45.
[15] N. Farina, M.G. Isaac, A.R. Clark, J. Rusted, N. Tabet, Vitamin E for Alzheimer's dementia and mild cognitive impairment (review), Cochrane Database Syst. Rev. 11 (2012), http://dx.doi.org/10.1002/14651858.
[16] A.J. Lees, J. Hardy, T. Revesz, Parkinson's disease, Lancet 373 (2009) 2055–2066.
[17] W.G. Meissner, M. Frasier, T. Gasser, C.G. Goetz, A. Lozano, P. Piccini, J.A. Obeso, O. Rascol, A. Schapira, V. Voon, D.M. Weiner, F. Tison, E. Bezard, Priorities in Parkinson's disease research, Nat. Rev. Drug Discov. 10 (2011) 377–393.
[18] F. Stocchi, C.W. Olanow, Neuroprotection in Parkinson's disease: clinical trial, Ann. Neurol. 53 (2003) S87–S99.
[19] K. Nakaso, C. Nakamura, H. Sato, K. Imamura, T. Takeshima, K. Nakashima, Novel cytoprotective mechanism of anti-parkinsonian drug deprenyl: PI3K and Nrf2-derived induction of antioxidative proteins, Biochem. Biophys. Res. Commun. 339 (2006) 915–922.
[20] M. Carbone, S. Duty, M. Rattray, Riluzole neuroprotection in a Parkinson's disease model involves suppression of reactive astrocytosis but not GLT-1 regulation, BMC Neurosci. 13 (2012) (doi: 1186/1471-2202-13-38).
[21] P. Hickey, M. Stacy, Adenosine A2A antagonists in Parkinson's disease: what's next? Curr. Neurol. Neurosci. Rep. 12 (2012) 376–385.
[22] T.A. Yacoubian, D.G. Standaert, Targets for neuroprotection in Parkinson's disease, Biochim. Biophys. Acta 1792 (2009) 676–687.
[23] K. Nakaso, S. Ito, K. Nakashima, Caffeine activates the PI3K/Akt pathway and prevents apoptotic cell death in a Parkinson's disease model of SH-SY5Y cells, Neurosci. Lett. 432 (2008) 146–150.
[24] Y. Numakawa, T. Numakawa, T. Matsumoto, Y. Yagasaki, E. Kumamaru, H. Kunugi, T. Taguchi, E. Niki, Vitamin E protected cultured cortical neurons from oxidative stress-induced cell death through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase, J. Neurochem. 97 (2006) 1191–1202.
[25] S. Takahama, T. Hirose, S. Ohno, aPLC restricts the basolateral determinant PtdIns (3,4,5)P3 to the basal region, Biochem. Biophys. Res. Commun. 368 (2008) 249–255.
[26] R. Comitato, K. Nesaretnam, G. Leoni, R. Ambra, R. Canali, A. Bolli, M. Marino, F. Virgili, A novel mechanism of natural vitamin E tocotrienol activity: involvement of ERβ signal transduction, Am. J. Physiol. Endocrinol. Metab. 297 (2009) E427–E437.
[27] K. Liang, L. Yang, C. Yin, Z. Xiao, J. Zhang, Y. Liu, J. Huang, Estrogen stimulates degradation of β-amyloid peptide by up-regulating neprilysin, J. Biol. Chem. 285 (2010) 935–942.
[28] Y. Berthois, J.A. Katzenellenbogen, B.S. Katzenellenbogen, Phenol red in tissue culture media is a weak estrogen: implications concerning the study of estrogen-responsive cells in culture, Proc. Natl. Acad. Sci. U. S. A. 83 (1986) 2496–2500.
[29] S. Khanna, S. Roy, H.A. Park, C.K. Sen, Regulation of c-Src activity in glutamate-induced neurodegeneration, J. Biol. Chem. 282 (2007) 23482–23490.
[30] X. Liu, N. Yamada, T. Osaswa, Assessing the neuroprotective effect of antioxidative food factors by application of lipid-derived dopamine modification adducts, Methods Mol. Biol. 594 (2010) 263–273.
[31] K. Fukui, H. Sekiguchi, H. Takatsu, T. Koike, T. Koike, S. Urano, Tocotrienol prevents AAPH-induced neurite degeneration in neuro2a cells, Redox Rep. 18 (2013) 238–244.
[32] F.M. Lopes, R. Schroder, M.L.C. da Fronta Jr, A. Zanotto-Filho, C.B. Muller, A.S. Piers, R.T. Meurer, G.D. Colpo, D.P. Gelain, F. Kapczinski, J.C.F. Moreira, M. da C. Fernandes, F. Klamt, Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson's disease studies, Brain Res. 1337 (2010) 85–94.
[33] M. Encinas, M. Iglesias, N. Llecha, J.X. Comella, Extracellular-regulated kinases and phosphatidylinositol 3-kinase are involved in brain-derived neurotrophic factor-mediated survival and neuritogenesis of the neuroblastoma cell line SH-SY5Y, J. Neurochem. 73 (1999) 1409–1421.
[34] J.K. Atwal, B. Massie, F.D. Miller, D.R. Kaplan, The Trk B-Shc site signals neuronal survival and local axon growth via MEK and PI3-kinase, Neuron 27 (2000) 265–277.
[35] Y. Mori, M. Higuchi, N. Masuyama, H. Gotoh, Adenosine A2A receptor facilitates calcium-dependent protein secretion through the activation of protein kinase A and phosphatidylinositol-3 kinase in PC12 cells, Cell Struct. Funct. 29 (2004) 101–110.
[36] V.D. Nair, S.C. Sealfon, Agonist-specific transactivation of phosphoinositide 3-kinase signaling pathway mediated by the dopamine D2 receptor, J. Biol. Chem. 278 (2003) 47053–47061.
[37] P. Mannella, R.D. Brinton, Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action, J. Neurosci. 26 (2006) 9439–9447.
[38] X. Sun, H. Yao, R.M. Douglas, X.Q. Gu, J. Wang, G.G. Haddad, Insulin/PI3K signaling protects dentate neurons from oxygen–glucose deprivation in organotypic slice cultures, J. Neurochem. 112 (2010) 377–388.
[39] E. Powell, E. Shanle, A. Brinkman, J. Li, S. Keles, K.B. Wisinski, W. Huang, W. Xu, Identification of estrogen receptor dimer selective ligands reveals growth-inhibitory effects on cells that co-express ERα and ERβ, PLoS One 7 (2013) e30993.
[40] G.G. Kuiper, E. Enmark, M. Pelto-Huikko, S. Nilsson, J.A. Gustafsson, Cloning of a novel receptor expressed in rat prostate and ovary, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 5925–5930.
[41] M. Marino, P. Galluzzo, P. Ascenzi, Estrogen signaling multiple pathways to impact gene transcription, Curr. Genomics 7 (2006) 497–508.
[42] T. Ivanova, P. Mendez, L.M. Garcia-Segura, C. Beyer, Rapid stimulation of the PI3-kinase/Akt signaling pathway in developing midbrain neurones by oestrogen, J. Neuroendocrinol. 14 (2002) 73–79.
[43] S. Chamniansawat, S. Chongthammakun, Genomic and non-genomic actions of estrogen on synaptic plasticity in SH-SY5Y cells, Neurosci. Lett. 470 (2010) 49–54.
[44] K.L. Chambliss, I.S. Yuhanna, R.G. Anderson, M.E. Mendelsohn, P.W. Shaul, ERβ has nongenomic action in caveolae, Mol. Endocrinol. 16 (2002) 938–946.
[45] H.P. Kim, J.Y. Lee, J.K. Jeong, S.W. Bae, H.K. Lee, I. Jo, Nongenomic stimulation of nitric oxide release by estrogen is mediated by estrogen receptor α localized in caveolae, Biochem. Biophys. Res. Commun. 263 (1999) 257–262.
[46] R.G. Parton, Caveolae and caveolins, Curr. Opin. Cell Biol. 8 (1996) 542–548.
[47] M. Marino, P. Ascenzi, Membrane association of estrogen receptor α and β influence 17β-estradiol-mediated cancer cell proliferation, Steroids 73 (2008) 853–858.
[48] J.I. Luoma, M.L. Boulware, P.G. Mermelstein, Caveolin proteins and estrogen signaling in the brain, Mol. Cell. Endocrinol. 290 (2008) 8–13.
[49] A.H. Schapira, C.W. Olanow, Neuroprotection in Parkinson disease: mysteries, myths and misconceptions, JAMA 291 (2004) 358–364.
[50] Y. Mizuno, N. Hattori, S. Kubo, S. Sato, K. Nishioka, T. Hatano, H. Tomiyama, M. Funayama, Y. Machida, H. Mochizuki, Progress in the pathogenesis and genetics of Parkinson's disease, Philos. Trans. R. Soc. Lond. B Biol. Sci. 363 (2008) 2215–2227.