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Original Article

Upregulation of cell surface estrogen receptor alpha is associated with the mitogen-activated protein kinase/extracellular signal-regulated kinase activity and promotes autophagy maturation

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Abstract: Recently, accumulating evidence has implicated the dysregulation of autophagy as underlying the pathophysiology of several neurodegenerative diseases. The human neuronal cell line SH-SY5Y was exposed to 1-Methyl-4-phenylpyridinium (MPP^+). The mechanism is that the sustained activation of the MAPK/ERK pathway by MPP^+ alters autophagy selectively at the maturation step, significant increasing in autophagy formation and delaying in autophagy degradation in SHSY5Y cells. In this study, we provided evidences that estrogen was capable of promoting SHSY5Y cells survival in MPP^+-treated group. In particular, the up-regulation of mERα, but not mERβ, was associated with a rapid and transient activation of ERK phosphorylation compatible with promoting autophagy maturation. The up-regulation of mERα changed the sustained activation of ERK phosphorylation in MPP^+-treated group into a temporary activation. Taken together, these findings strongly support that the expression of mERα promotes the maturation of autophagosomes into functional autolysosomes by regulating ERK, determining SHSY5Y cells survival.

Keywords: Surface estrogen receptor alpha, ERK, autophagy maturation

Introduction

Parkinson's disease is a neurodegenerative disorder characterized by the progressive loss of dopaminergic neurons of the nigrostriatal pathway in the brain [1]. Another characteristic of PD is its late onset and progressive nature. Environmental toxins MPP^+ (1-methyl-4-phenylpyridinium) can directly inhibit axon transport in the squid axoplasm and DA neurons, and interfere with oxidative phosphorylation in mitochondria, causing depletion of ATP and cell death. It also inhibits the synthesis of catecholamines, reduces levels of dopamine and cardiac norepinephrine [2]. Autophagy is an evolutionarily conserved catabolic process by which the cell recycles or degrades proteins or damaged cytoplasm organelles. Autophagy involves the sequestration of cellular components within a membrane, the so-called 'autophagosome'. Autophagosomes then fuse with embosomes or directly with lysosomes to form autolysosomes, resulting in the degradation of their components by hydrolytic enzymes [3]. As a result, autophagy controls cell modeling throughout development and prevents cell aging during life. Not only being a housekeeping process, autophagy protects the cells against the accumulation of damaged organelles and genotoxic substances that would otherwise induce mutations.

As known, autophagy plays an important role in the pathogenesis of some diseases, especially in the Parkinson's disease (PD). But, autophagy could be interfered by abnormal activation of the phosphatidyl-inositol 3-kinase pathway, the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway, and two well-known estrogen-activated signaling cascades [4-7]. Several lines of evidence indicate that 17β-estradiol (E2) has been implicated to be neuroprotective against a variety of neurodegenerative disorders [8]. There is a cel-

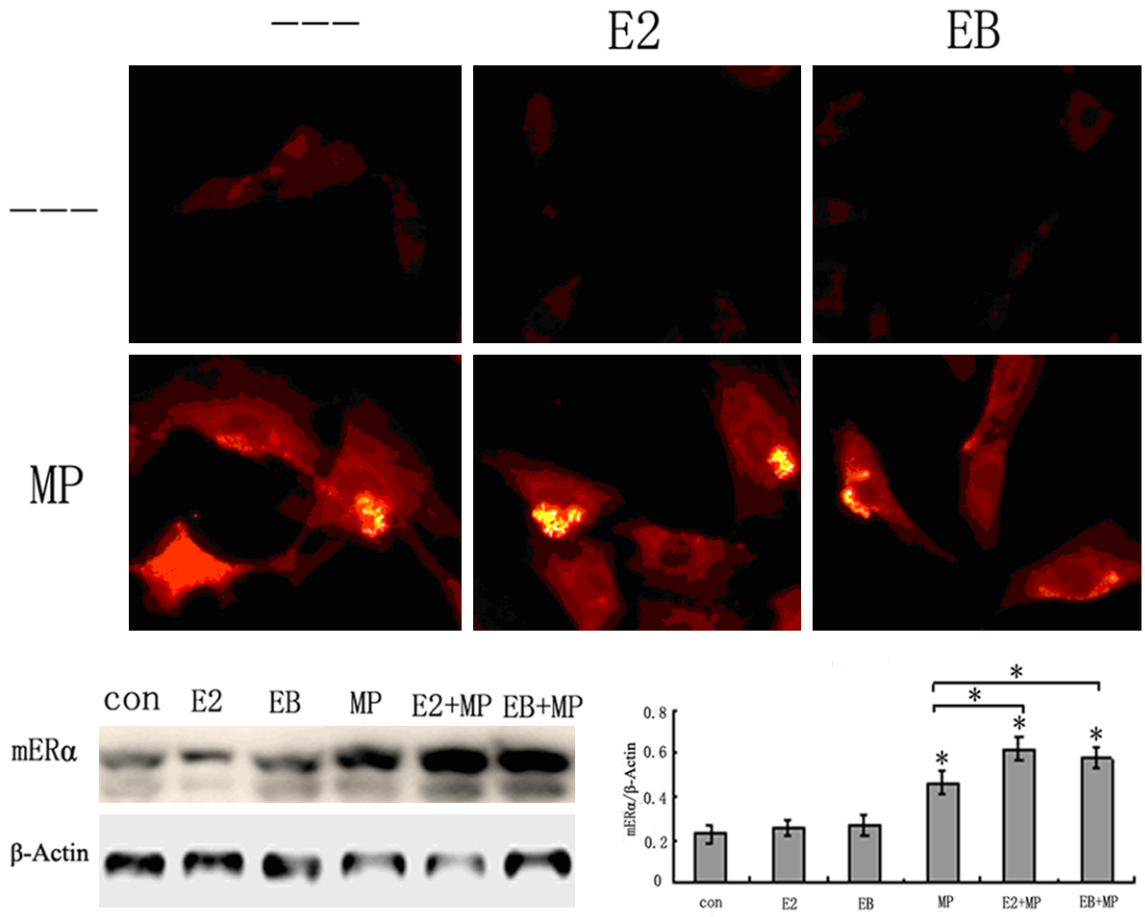

Figure 1. Immunofluorescence analysis of cell surface mERα in Triton ×100 unpermeated SH-SY5Y cells. SHSY5Y cells were cultured in the presence of MPP^+ (MP, 5 μg/mL), E2, E2BSA (E2, EB, 10 nM), or not. In 24 h, cells were observed by fluorescence microscopy (Magnification: ×300). mERα expression (red fluorescence) in Triton ×100 unpermeated SH-SY5Y cells. Note mERα expression (red spots) suggesting a colocalization of ERα in E2 or E2BSA/MPP^+ exposed cells. No red spots are instead observable in untreated cells. The expressions of mER in membrane proteins were analyzed by Western blotting. Densitometric analysis of mER levels relative to β-actin is also shown. Values are expressed as means ± SD from 10 independent experiments. $^*P<0.05$.

lular and molecular basis for these clinical observations. The primary mechanism of E2 neuroprotection in the brain is mediated principally by the "classical" nuclear ER-mediated genomic signaling pathway, which involves E2 interaction with nuclear ER (nER), ERα and ERβ (ER alpha and ER beta), to produce genomic effects exerting the cytoprotective effects. Along these lines, several studies have shown that nER regulates Ca^{2+} dynamics and increases the expression of the anti-apoptotic gene, bcl-2, and the antiapoptotic prosurvival factor in hippocampal neurons [9-11]. In addition to genomic signaling, there is increasing evidence that rapid nongenomic signaling via membrane localized extranuclear ER (mERα and mERβ) may also play a role in mediating E2 neuroprotective effects in the brain. Multiple laboratories have indicated that estrogen activation of membrane sites of action results in regulation of calcium responses and activation of various protein kinase cascades, including extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), protein kinase A, protein kinase C, Akt and phosphatidylinositol 3-OH kinase (PI3K) [12, 13]. For instant, it has been suggested that the upregulation of nonnuclear mERα mediates the rapid attenuation of ATP-induced $[Ca^{2+}]i$ signaling in mouse dorsal root ganglion neurons [14]. Several laboratories have shown that the rapid activation of extracellular signal-regulated kinases 1/2 (ERKs) by E2 is critical for its neuroprotective effects [15-17]. In addition, under chronic minimal perox-

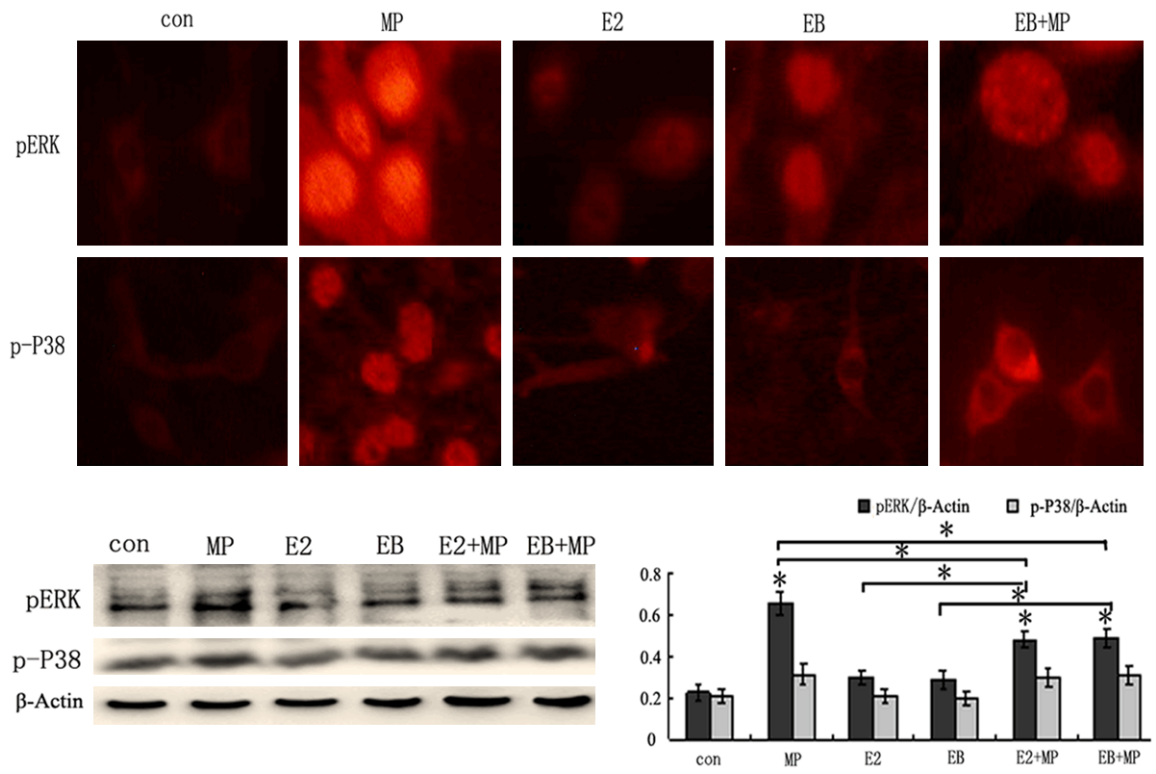

Figure 2. Effect of E2BSA and E2 on the activation of ERK and p38 MAPK. SHSY5Y cells were cultured in the presence of MPP^+ (MP, 5 μg/mL), E2, E2BSA (E2, EB, 10 nM), or not. In indicating time, cells were observed by fluorescence microscopy (Magnification: ×300). In physiological conditions, estrogen (either E2BSA or E2) increased ERK phosphorylation (pERK) slightly. Conversely, under MPP^+ pretreatment 12 h, both non-permeant and permeant forms of E2 significantly increased pERK levels. Densitometric analysis of p-ERK1/2 and p-P38 levels relative to β-actin is also shown by Western blot analysis. Values are expressed as means ± SD from 10 independent experiments. $^*P<0.05$.

ide treatment, upregulation of mERα, not mERβ, might trigger ERK phosphorylation and dephosphorylation of P38, regulating autophagy and promote neuronal survival [18]. In particular, it is cell survival through ER alpha non-genomic signaling and cell death through ER beta non-genomic signaling [19]. However, the mechanisms underlying these protective effects are still under investigation, especially the role in PD and the mechanism how to regulate autophagy.

The aim of this study was to clarify the role of ER in determining neuronal cell fate and the possible implication of ER receptors in regulating autophagy in an in vitro model of Parkinson's disease, SH-SY5Y cells treated with MPP^+. The results of our study revealed that estrogen decreased cell vacuolation by promoting the maturation of autophagosomes into functional autolysosomes in MPP^+-treated group. The activation of MAPK/ERK is essential for MPP^+ or estrogen to induce vacuolation, but has opposite effects. Interestingly, the activation of MAPK/ERK does not regulate the autophagy initiation step but rather the maturation step. The study provides important evidences that cell surface ER receptor activation plays the important role in estrogen-induced neuroprotection and suggests that ERK signaling is an important component mediating extra nuclear estrogen receptor beneficial neural effects. Most importantly, we provide the first evidence that upregulation of mERα, not mERβ, mediate the temporary and significant mitogen-activated protein kinase/extracellular signal-regulated kinase activity and promote autophagy maturation in an *in vitro* model of Parkinson's disease.

Materials and methods

Cell culture and treatments

The mouse SHSY5Y cell line was maintained in DMEM containing 10% FBS. For all the exper-

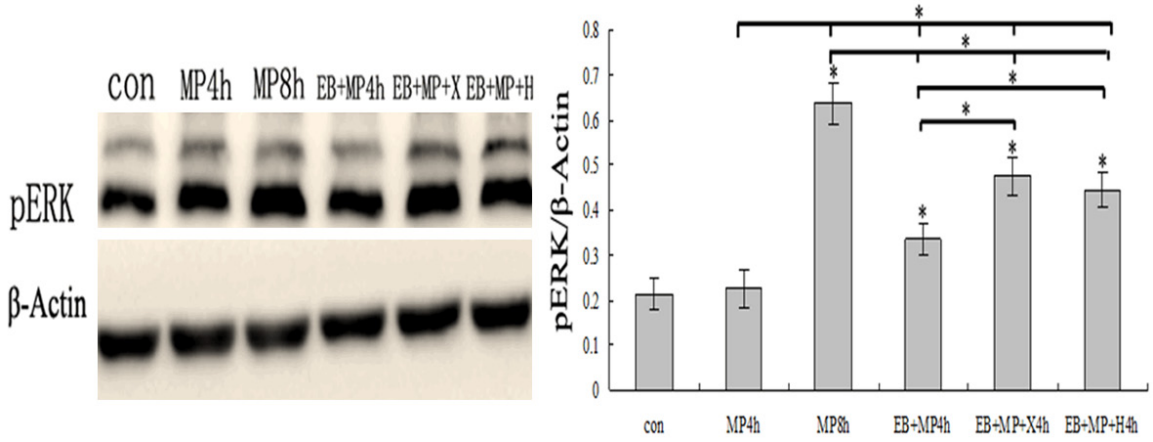

Figure 3. The time course of activation of the MAPK/ERK pathway. SHSY5Y cells were stimulated with E2BSA (EB, 10 nM), MPP^+ (MP, 5 μg/mL), XCT790 (×, 5 mg/ml), 5-Hydroxytamoxifen (H, 2 mg/mL) or vehicle, and lysed at indicated times and analyzed for ERK activation by Western blotting. Blot shown is representative of 3 independent experiments. Densitometry analysis of pERK levels relative to β-actin is shown. Values are expressed as means ± SD from 10 independent experiments. $^*P<0.05$.

iments, cells were serum starved for 2 in fresh DMEM supplemented with 0.1% bovine serum albumin (BSA A7030, Sigma) and treated with MPP^+ (5 μg/mL; Sigma) alone or in combination with E2 (sigma) and with membrane-non-permeant E2BSA (molar ratio E2 : BSA = 30:1; Sigma) at a concentration of 10 nM (physiological concentration) for 24 h or indicating times. When either XCT790 (5 mg/mL; sigma; an ERα inverse agonist), 5-Hydroxytamoxifen (2 mg/mL; sigma; an ER inhibitor), or bafilomycin A1 (100 nmol/L; Sigma; an inhibitor of fusion between autophagosomes and lysosomes), MEK1/2 inhibitor U0126 (10 mmol/L) was used, it was added to the starvation medium for 30 minutes before the addition of estrogen. As controls, cells were incubated with vehicle (Me2SO; 1:1,000), chloroquine (500 μmol/L, Sigma). The effects of estrogen on the autophagy pathway were studied by transmission electron microscopy, LC3-aggregation, and biochemical assays.

Immunofluorescence staining

Immunofluorescent staining was performed as described previously [15]. The cells were fixed with 4% formaldehyde in PBS at 37°C for 10 min, and then incubated for 24 h at 4°C with a primary antibody diluted in PBS containing 0.2% Triton X-100 or not. Here, the primary antibodies were mouse anti-pERK antibody (1:1000; Chemicon), rabbit anti-LC3 antibody (1:1000; Abgent), mouse anti-ERα antibody (1:1000; Abcam), and mouse anti-ERβ antibody (1:1000; Santa Cruz). Subsequent incubations were performed at room temperature with a secondary antibody [anti-rabbit cy3 (1:300) or anti-mouse cy2 (1:200); Sigma] for 1 h. Then, the cells were counterstained with DAPI (1:1000; Sigma) and mounted. Before every protocol, cells were rinsed 3 times with 0.01 M PBS, each time for 5 min. For visual enumeration of the immunostained cells in the cultures, five representative areas per well of the 24-well plate were counted under the fluorescence microscope. Results were obtained by three independent individuals in a blinded manner.

Lysosomal enzyme activities

SH-SY5Y was treated with different factors. After 24 hours, cells were washed, scraped, and homogenized by sonication in water. Lysosomal enzyme activities were detected using fluorometric and colorimetric assays as described [16]. In each experiment, the enzyme activities were measured from two parallel culture dishes.

Western blotting

Cell lysates were prepared in RIPA lysis buffer and analyzed by Western blotting as previously described [12] with antibodies including mouse anti-phospholyrated-ERK1/2 (pERK1/2) pAbs, rabbit anti-LC3 pAbs and anti mouse anti-β-actin monoclonal Ab (sigma). The membrane proteins were analyzed with rabbit anti-ERα pAbs and mouse anti-ERβ pAbs (1:1000; Cell

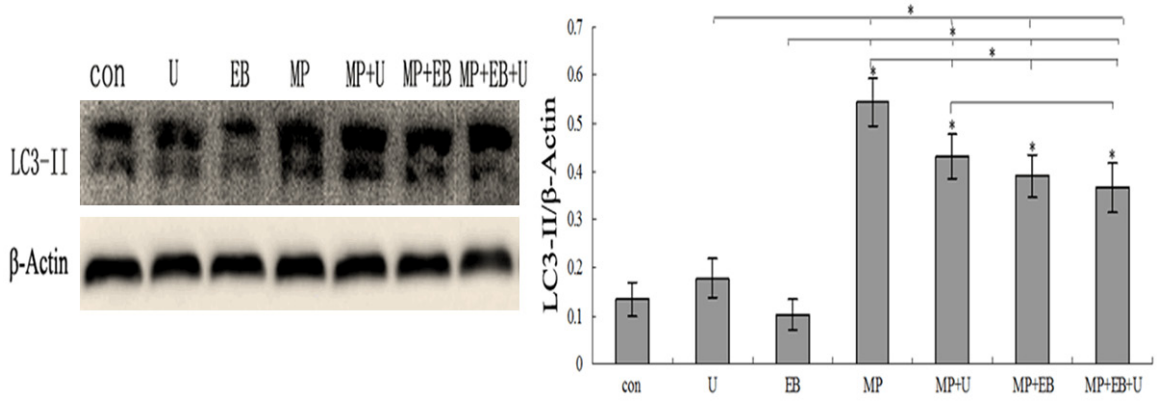

Figure 4. Effect of E2BSA and E2 on the modulation of autophagy. SHSY5Y cells were stimulated with E2BSA (EB, 10 nM), MPP^+ (MP, 5 μg/mL), U0126 (U, 10 mmol/L) or vehicle, and lysed at 16 h, and western blot analysis of LC3-II in untreated and MPP^+ treated SH-SY5Y cells in presence or absence of E2 or E2BSA. Blots shown are representative of 10 independent experiments. Densitometry analysis of LC3-II levels relative to β-actin is shown. Values are expressed as mean ± SD from 10 independent experiments. $^*P<0.05$.

Signaling Technologies). Proteins were quantified by densitometry analysis of the autoradiograms (GS-700 Imaging Densitometer, Bio-Rad). To see if accumulation of LC3-II is due to increased autophagosome formation or impaired autophagosome-lysosome fusion, LC3-II is assessed in the presence of bafilomycin A1, that blocks autophagosome-lysosome fusion [20], as shown previously [13, 21].

Electron microscopy

The ultrastructural changes caused by estrogen or MPP^+ in SHSY5Y were analyzed by transmission electron microscopy as previously described [13]. From each sample were randomly taken 20 to 25 micrographs (primary magnification, 10,000). Then, the volume of autophagic vesicles was estimated by morphometry using Visilog program (Leica TCS SP2 CLSM, School of Medicine Electron Microscopy Center, Fudan University). Autophagic vesicles were classified as early, containing morphologically intact cytoplasm, and late, containing partially disintegrated and electron dense material [14].

Statistical analysis

All data was collected from a minimum of three independent experiments done in triplicate. The significance of effects between control and experimental conditions was determined by a Student t test or one-way ANOVA with Bonferroni Multiple Comparisons tests. Statistical significance was set at $P<0.05$.

Results

MPP^+ induces up-regulation of surface ERα, and increases the effect of estrogen

On the basis of literature demonstrating the presence of functional ER at the surface membrane of cells of different histotypes [22-25], we evaluated the cell surface expression of ER in Triton X100 unpermeated SH-SY5Y cells. By immunofluorescence microscopy, we first investigated the expression of mER in physiological condition. This analysis revealed that both mERα and mERβ were undetectable on SH-SY5Y untreated cells (**Figure 1**). Conversely, under MPP^+-induced stress, mERα expression was observed on SH-SY5Y cells (**Figure 1**). Increased expression of mERα could be induced by a membrane permeant E2 form (E2) or an embrane non-permeant form of E2 (E2BSA). Consistent with the results obtained by immunofluorescence microscopy, western blotting analysis clearly showed that increased expression of ERα in cell membrane proteins could be induced by E2 or E2BSA, but lower than the frequency of MPP^+ induction. Combined with E2 or E2BSA, ERα expression increased further (**Figure 1**); but ERβ expression didn't change obviously (data not shown).

mER regulated activation of the ERK, but not p38

Based on the results reported above, an important point was to evaluate whether mER were functional receptors. To provide evidence of a

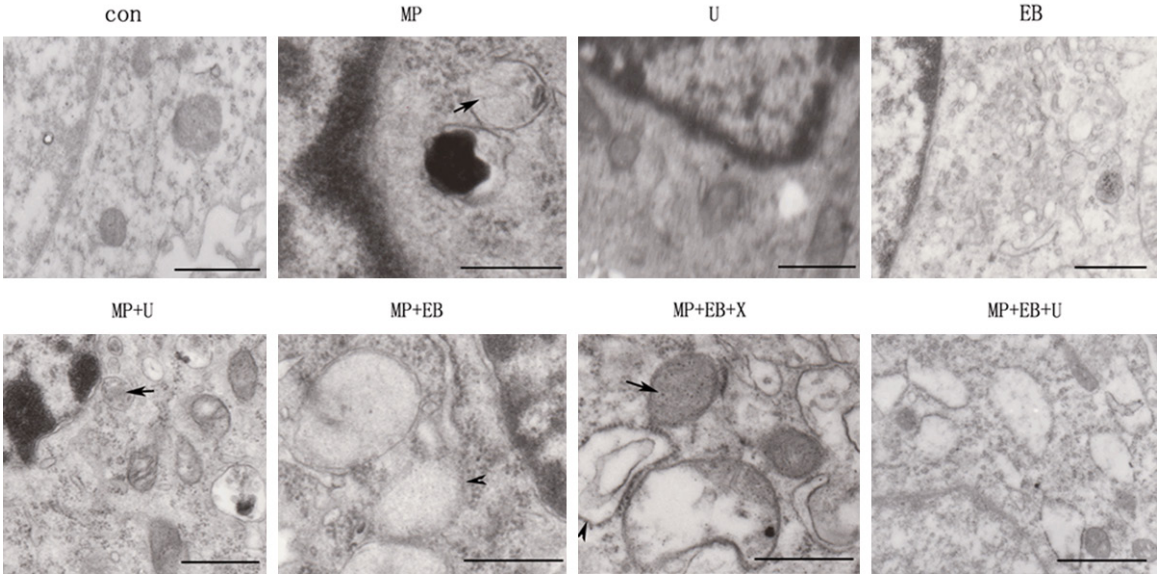

Figure 5. E2BSA promotes the improvement of giant autolysosomal compartment in MPP^+-treated group. SHSY5Y cells were stimulated with E2BSA (EB, 10 nM), MPP^+ (MP, 5 μg/mL), XCT790 (×, 5 mg/ml), U0126 (U, 10 mmol/L) or vehicle. In 16 h, cells were observed by electron microscopy (bar 500 nm). Numerous large autophagic vesicles were observed within MPP^+-treated cells but not in untreated cells, in which cell mitochondria swelled, the crest of mitochondria reduced, the endoplasmic reticulum expanded, the nuclei shrank, and the chromatin aggregated at the edge of the nucleus. The shape of mitochondria appeared unaffected with well defined cristae at proximity of autophagosomes in E2BSA-treated cells. E2BSA decreased the amount of giant autophagosomes in MPP^+-treated cells, and the middle-size autophagosomes kept unchange, suggesting that they were formed by the increased fusion of autophagosomes.

signaling function for estrogen via mER on neuronal cells, we determined in SH-SY5Y cells the effect of E2BSA and E2 on the activation of two key molecules involved in protein kinase cascades regulating cell homeostasis: ERK and p38 MAPK. We found that in physiological conditions, estrogen (either E2BSA or E2) increased ERK phosphorylation (pERK) slightly (**Figure 2**), but no significant difference. Under MPP^+-induced stress, significant increased pERK was observed. Conversely, combined with MPP^+, both non-permeant and permeant forms of E2 significantly increased pERK levels, but the levels of pERK were lower than that in MPP^+-treated group (**Figure 2**). Regarding p38 phosphorylation (p-p38), treatment with both non permeant and permeant forms of E2 did not influence p-p38 level. Interestingly, E2BSA exposure (30 min before MPP^+) was capable of preventing the increase of p-p38 induced by MPP^+ treatment (**Figure 2**).

Time course experiments were performed to characterize further the relationship between ERK activation and the estrogen response. Increased phospho-ERK was observed beginning ~8 hours after MPP^+ administration, While ~4 hours after combining E2BSA and MPP^+ administration (**Figure 3**). Moreover, MPP^+ induced remarkable growth of pERK, while combining MPP^+ and E2BSA induced moderate activation of the ERK. Therefore, we suggested that estrogen accelerate the speed of the activation of ERK pathway, but cut its amplitude. XCT790 could lower down the effect of E2BSA on ERK signal pathway, but 5-hydrotamoxifen couldn't increase the efficacy of XCT790 (**Figure 3**).

mERα modulates autophagic maturation by ERK signal way

MPP^+ increasing LC3-II shift was observed by 16 hours. E2BSA had no effect on the expression of LC3-II, but could downregulate the expression of LC3-II in group treated by MPP^+. Meanwhile, U0126 could downregulate the expression of LC3-II in MPP^+-treated group, too. However, if the U0126 was added >20 hours after MPP^+ treatment, the expression of LC3-II was not significantly different from groups treated with MPP^+ alone (**Figure 4**). MPP^+ induced the sustained activation of ERK1/2 and

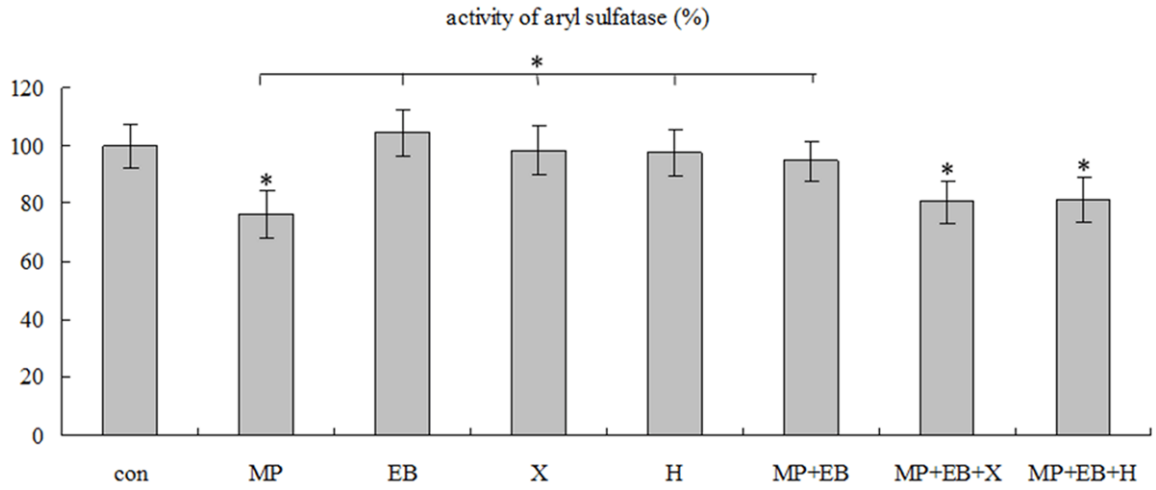

Figure 6. E2BSA regulated lysosomal activity of aryl sulfatase A in MPP⁺-treated cells. SHSY5Y cells were cultured in the presence of MPP⁺ (MP, 5 μg/mL), E2BSA (EB, 10 nM), MPP⁺ (MP, 5 μg/mL), XCT790 (×, 5 mg/ml), U0126 (U, 10 mmol/L) or not for 24 h. Lysosomal enzyme activities were detected using fluorometric and colorimetric assays. Values are expressed as mean ± SD from 10 independent experiments. $^{*}P<0.05$.

abrogation of this activation by pretreatment with the specific MEK1/2 inhibitor U0126 (10 mmol/L) significantly decreased MPP⁺-induced vacuolation. E2BSA induced the temporary activation of ERK1/2 in groups combined E2BSA and MPP⁺, and abrogation of this activation with XCT790 could increase the ratio of autophagosmes/autolysomes, but 5-hydrotamoxifen couldn't (**Figure 5**).

Upregulated mERα could improve lysosomal enzyme activities

Reduced lysosomal enzyme activities could be involved in the compromised autophagic degradation in MPP⁺-treated cells. MPP⁺ decreased the intracellular activity of aryl sulfatase A by 19.8 ± 2.3% compared with control values. Combined with E2BSA, the number of giant autophagosomes reduced and the activity of lysosomes rose. That is to say, these data point to a decline of lysosome hydrolase activity in MPP⁺-treated cells, thus reducing autophagic degradation. upregulated mERα could improve lysosomal enzyme activities, and induce autophagic maturation (**Figure 6**).

mERα promoted maturation of autophagy in MPP⁺-treated group

In order to explore the effect of mERα on maturation of autophagy, we draw into bafilomycin A1, an inhibitor of fusion between autophagosomes and lysosomes [20]. We found that bafilomycin A1 increased the expression of LC-3 II in E2BSA-treated group by 27.9%, while bafilomycin A1 only increase the expression of LC-3 II in MPP⁺-treated group by 2.7%. Moreover, when the cells in MPP⁺-treated group were pretreated with E2, bafilomycin A1 increased the expression of LC-3 II by 15.7%. Bafilomycin A1 also promoted the appearance of giant autophagic vacuoles in estrogen-treated group (**Figure 7**). Inhibition of the fusion was reversible, and the autophagosomes changed into autolysosomes after the removal of the inhibitor.

Discussion

Increasing evidences have suggested that estradiol exerts neuroprotective effects in a variety of neurodegenerative disorders, including stroke, Alzheimer's disease and Parkinson's disease. The primary mechanism of E2 neuroprotection in the brain is mediated by the classical estrogen nuclear receptors. In addition to the classical nuclear estrogen receptor, the expressions of non-nuclear estrogen receptors localized to the cell surface membrane (mER) have recently been demonstrated. Many investigators have subsequently contributed to this area, but have little defined key aspects of functions of mER, especially in PD. In this study, we showed that estrogen promoted cells survival, in an *in vitro* model of Parkinson's disease, SH-SY5Y cells treated with MPP⁺. Importantly, it

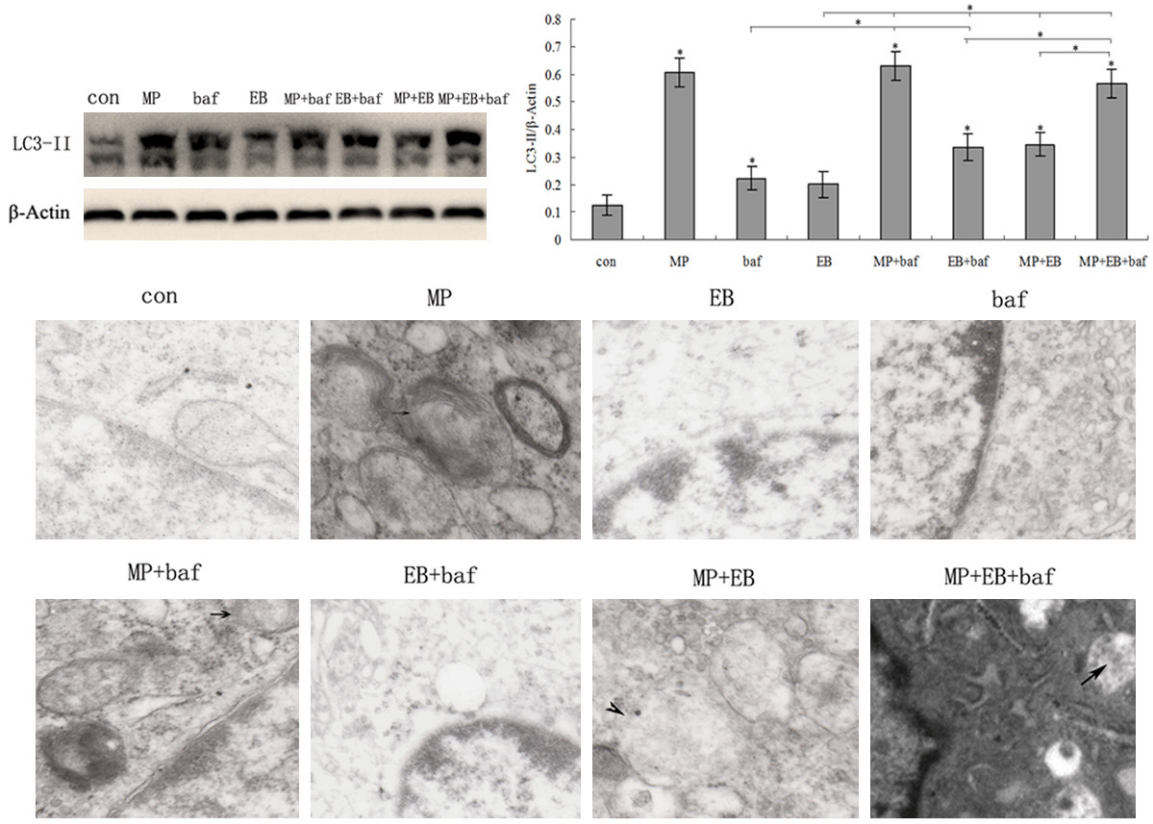

Figure 7. E2BSA promoted maturation of autophagy in MPP^+-treated groups. Cells were pretreated with E2BSA (EB, 10 nM), MPP^+ (MP, 5 μg/mL), bafilomycin A1 (baf, 100 nmol/L), or vehicle for 24 h. At the indicated times, cells were lysed, and maturation of autophagy was assessed by electron microscopy (bar 500 nm) and Western blotting with mouse anti-LC3 antibody (1:1,000). Bafilomycin A1 increased the expression of LC-3II in E2BSA-treated group by 27.9%, and only 2.7% in MPP^+-treated group. Values are expressed as mean ± SD from 10 independent experiments. $^*P<0.05$. Bafilomycin A1 also promoted the appearance of giant autophagic vacuoles in estrogen-cotreated group. Inhibition of the fusion was reversible, and the autophagosomes could change into autolysosomes after the removal of the inhibitor.

was the upregulation of mERα, not mERβ, at the cell surface associated with the he temporary and significant activation of ERK and promoted autophagy maturation. The abrogation of these effects by pretreatment with a mERα antagonist indicated the involvement of mERα in the neuroprotective mechanism mediated by estrogens.

Compelling evidence suggests that dysregulation of autophagy results in the accumulation of abnormal proteins and/or damaged organelles, which is commonly observed in neurodegenerative diseases, such as Alzheimer, Huntington's, and Parkinson's diseases (PD) [26]. Targeting autophagy has been therefore proposed as one strategy for treating PD. However, this therapeutic promise should uncover the underlying signaling pathways of autophagy. Several studies have showed that autophagy is regulated by activation of the mitogen activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) pathway and two well-known estrogen-activated signaling cascades [5-7]. Meanwhile, numerous studies have showed that the mER could bind ligand to activate various protein kinase cascades, including extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK), protein kinase A, protein kinase C, Akt, and phosphatidylinositol 3-OH kinase (PI3K) [13]. And the activation of the MAPK/ERK regulates the maturation of autophagosomes [20]. Based on the above studies showing that ERK signaling may be an important link between autophagy and mER regulation and regulates the autophagy maturation step.

The present study provided the evidence that the SHSY5Y cells responded *in vitro* directly to

MPP^+ by promoting extensive vacuolation and the sustained activation of the MAPK/ERK pathway consistent with our previous findings. However the sustained activation of the MAPK/ERK pathway by MPP^+ alters autophagy selectively at the maturation step, not the initial step, disrupting the maturation of autophagosomes into functional autolysosomes (i.e., significant increasing in autophagy formation and delaying in autophagy degradation) and results in SHSY5Y cells death [22]. On the contrary, the transient activation of the MAPK/ERK pathway induced by mER promoted SHSY5Y cells survival in this study. The expression levels of mERα and mERβ, two different mER isoforms, have been demonstrated to having different effects in regulating cell survival and death. In fact, an increased expression of mERα has to be considered as protective for cell survival whereas an increased expression of mERβ is for cell death [19]. In this study, we found that mERα, not mERβ, was upregulated at the surface of SHSY5Y cells in MPP^+-treated group. This could be a protective responding to MPP^+, relevant in the maintenance of cell homeostasis. Meanwhile, changes in the expression level of mERα, not mERβ, was consistent with increase or decrease of pERK expression in this study. Pretreatment with the ERα inverse agonist XCT790 decreased the expression level of mERα and pERK, and abrogated autophagic vacuolation inducing the repetitive sequestration of autophagosomes into lamellar bodies. This suggested that mERα was associated with the ERK signaling in our study. It was the upregulation of mERα increased ERK phosphorylation and mediated the E2 induced neuroprotective effects in the MPP^+-treated group. Interestingly, the ER antagonist 5-Hydroxytamoxifen was not able to block further estradiol-induced phosphorylation of ERK in this study, further excluding the involvement of the extracellular estrogen receptor-β [24].

These data created an apparent paradox in that MPP^+, mER and growth factors all regulate ERK and yet have opposite effects on autophagy [25]. The sustained activation of ERK by MPP^+ disrupts the maturation of autophagosomes into functional autolysosomes, while the transient activation of ERK by mERα promoted maturation of autophagosomes. An attractive possibility to explain this divergence might be to consider that the cell response depends critically of the nature, strength, and duration of ERK pathways activated [27]. The role of ERK in regulating the autophagy maturation step gives a further understanding of the mERα-induced neuroprotection in PD. In addition to some literature data [28, 29], our date are first to document that it is upregulation of mERα associated with the mitogen-activated protein kinase/extracellular signal-regulated kinase activity and promotes autophagy maturation, leading to cell survival.

Disclosure of conflict of interest

None.

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