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Paeoniflorin, a potent natural compound, protects PC12 cells from MPP+ and acidic damage via autophagic pathway

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ABSTRACT

Ethnopharmacological relevance: Paeoniflorin (PF) is the principal bioactive component of *Radix Paeoniae alba*, which is widely used in Traditional Chinese Medicine for the treatment of neurodegenerative disorders such as Parkinson's disease (PD).
Aim of the study: To evaluate the neuroprotective effects of PF on MPP+- or acid- (pH 5.0) induced injury in cultured PC12 cells and to investigate the activity of autophagy-lysosome pathway (ALP). Amiloride (Ami), a non-selective blocker of acid-sensing ion channels (ASICs), as a positive control drug, since it is neuroprotective in rodent models of PD.
Materials and methods: The cell viability was analyzed with MTT assay. The cell injury was assessed by lactate dehydrogenase (LDH) assay. Flow cytometry and Western blot analysis were used to study the apoptotic, calcium influx and autophagic mechanisms.
Results: Ami (100 μM) and PF (50 μM) both protected PC12 cells against MPP+- or acid-induced injury as assessed by MTT assay, lactate dehydrogenase release, and apoptosis rate. The concentrations of cytosolic free Ca^{2+} were raised after exposure to MPP+ or acidosis, while Ami and PF both reduced the influx of Ca^{2+}. More importantly, we found that the mechanisms of neuroprotective effects of Ami and PF were closely associated with the upregulation of LC3-II protein, which is specifically associated with autophagic vacuole membranes. Furthermore, application of MPP+ or acid induced the overexpression of LAMP2a, which is directly correlated with the activity of the chaperone-mediated autophagy pathway. However, Ami and PF inhibited the overexpression of LAMP2a.
Conclusions: Our data provide the first experimental evidence that PF modulates autophagy in models of neuron injury, as well as providing the first indication of a relationship between ASICs and ALP.

1. Introduction

Peoniflorin (PF), a monoterpene glycoside isolated from the aqueous extract of the Chinese herb *Radix Paeoniae alba*, possesses wide pharmacological effects in the nervous system. It has been used in the treatment of cerebral ischemia (Liu et al., 2006), epilepsy (Tsuda et al., 1997), and neurodegenerative disorders such as Alzheimer's disease (Zhong et al., 2009) and Parkinson's disease (Liu et al., 2007). While the mechanisms of the neuroprotective effects of PF are still unclear, accumulating evidence suggests that it is closely associated with the homeostasis of mitochondria, including the suppression of inflammatory reactions (Liu et al., 2006), maintenance of intracellular calcium concentration (Tsai et al., 2005) and its ability to scavenge for reactive oxygen species (ROS) (Li et al., 2007), which lead to a progressive accumulation of oxidized protein aggregates and of dysfunctional mitochondria. The autophagy-lysosome pathway (ALP) is considered to be the main responsible mechanism for the removal of misfolded pro-

Abbreviations: PF, paeoniflorin; PD, Parkinson's disease; ALP, autophagy-lysosome pathway; LC3, microtubule associated protein light chain 3; LAMP2a, receptor lysosome-associated membrane protein 2a; Ami, amiloride; ASICs, acid-sensing ion channels; MPP+, 1-methyl-4-phenylpyridinium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; ROS, reactive oxygen species; CMA, chaperone-mediated autophagy; MPTP, 1-metryl-4-phenyl-1,2,3,6-tetrahydropyridine; LDH, lactate dehydrogenase; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide; AV, annexin V 488; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; TBS, Tris-buffered saline; TBST, TBS containing 0.05% Tween-20; HRP, horse radish peroxidase; ECL, enhanced chemiluminescence; SD, standard deviation; ANOVA, analysis of variance; UPS, ubiquitin-proteasome system; PICK1, protein interacting with C-kinase 1.

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teins and cellular debris including mitochondria (Zhang and Ney, 2009), thus the role of autophagy as a survival strategy of neurons constitutes an emerging topic in the study of the pathogenesis of neurodegenerative disorders. Given that the neuroprotective effects of PF are tightly associated with the homeostasis of mitochondria, we hypothesized that PF modulates the activity of ALP for the clearance of misfolded proteins and dysfunctional mitochondria.

It is well known that ALP is composed of macroautophagy (generally referred to as autophagy), microautophagy, and chaperone-mediated autophagy (CMA) that differ in physiological function and delivery mode to lysosomes (Pan et al., 2008). Autophagy can be induced within short periods of nutrient deprivation and is the only mechanism by which entire organelles such as mitochondria are recycled (Pan et al., 2008); CMA is a secondary response that temporally follows autophagy (Pan et al., 2008) and is a selective pathway for the degradation of oxidized and damaged proteins (Kaushik and Cuervo, 2006); while microautophagy is not activated by nutritional deprivation or stress (Pan et al., 2008). So, in this study, we focused on the activities of autophagy and CMA by using specific proteins: microtubule associated protein light chain 3 (LC3-II) and receptor lysosome-associated membrane protein 2a (LAMP2a) respectively. LC3-II binds to the membrane of autophagic vacuoles and regulates their formation in cells. To date, LC3-II is the best identified mammalian protein species specifically associated with autophagic vacuole membranes (Komatsu et al., 2007). In CMA, the limiting step is the interaction of the substrate-chaperone complex with LAMP2a, so CMA activity is direct correlated with levels of LAMP2a in the lysosomal membrane (Kaushik and Cuervo, 2006).

Parkinson's disease (PD) is one of the most common neurodegenerative diseases in the elderly. Although the etiology of this disease remains elusive, post-mortem analyses of PD brains suggest that mitochondrial dysfunction is evident in the brains of PD patients (Schapira, 2008). 1-Methyl-4-phenylpyridinium (MPP^+), the active metabolite of 1-metryl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), has been used extensively in various mammalian species to produce an experimental model of PD. MPP^+ produces mitochondria-targeted injury (Esteves et al., 2008), resulting in oxidative stress (Chen et al., 2009) and culminating in the loss of dopaminergic neurons (Ourednik et al., 2009). Recent studies demonstrate that MPP^+ also alters the function of autophagy (Chu et al., 2007). On the other hand, acidosis is a common cause of mitochondrial dysfunction and neuron degeneration as a consequence of pathological activation of acid-sensing ion channels (ASICs). This is especially true in the activation of ASIC1a, the dominant subtype in the central nervous system, which conducts calcium, and calcium overload is recognized as a final common pathway in neurodegenerative disorders (Yermolaieva et al., 2004). Although there is no directly evidence of an association between ASICs and autophagy, the diuretic amiloride (Ami), a non-selective blocker of ASICs, has been reported to protect substantia nigra neurons in MPTP models of PD (Arias et al., 2008). So in this study, we exposed PC12 cells (a rat pheochromocytoma cell line) to two different media, MPP^+ and acidic medium of pH 5.0, both having the capacity to damage mitochondria. At the same time we used Ami as a positive control drug.

In this work, we used different approaches to evaluate the neuroprotective effects of Ami and PF, and investigated the changes of specific proteins of autophagy and CMA by Western blot analysis. Our data provide the first experimental evidence of PF modulation of autophagy in models of neuron injury, and provides the first indication of a relationship between ASICs and ALP.

2. Materials and methods

2.1. Cell culture and treatments

A rat pheochromocytoma (PC12) cell line was purchased from Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). PC12 cells were cultured in 25 cm^2 culture flasks at 37 °C under an atmosphere of 5% CO_2/95% air in RPMI-1640 medium (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) and 1% penicillin-streptomycin. For the experiments, the cells were detached and re-seeded either in 96-well plates (1.0×10^4 per well) or 6-well plates (8.0×10^5 per well). Next day, the cultures were treated with either MPP^+ (0.5–1.0 mM) or acidic medium at pH 5.0 for 24 h. Some cultures also received Ami (amiloride hydrochloride hydrate, Sigma–Aldrich, USA) or PF (Paeoniflorin, purchased from National Institute for the Control of Pharmaceutical and Biological Products of China, Beijing). Cells cultured in RPMI-1640 medium were used as vehicle control. After the treatment, the cells cultured in 96-well plates were used for MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma–Aldrich) assay and those in 6-well plates were used for lactate dehydrogenase (LDH) assay, flow cytometry, and Western blot analyses.

2.2. MTT assay for cell viability

Cell viability was measured by MTT assay as described in a reference (Lee et al., 2008). Cells from at least six wells were used for each group. Briefly, 24 h after incubation in the conditioned medium, the supernatant was removed and the cells were gently washed with PBS followed by 80 μl fresh culture medium and addition of 20 μl MTT (5 mg/ml, Sigma–Aldrich). After 2 h incubation at 37 °C, the supernatant was removed and the intracellular formazan product was dissolved in 150 μl DMSO with shaking for 10 min. Absorbance was measured at 570 nm with an ELISA reader (Model 680, Bio-Rad, USA). Cell viability was expressed as a percentage of the value against the control group.

The conditioned medium was established after the MTT cell viability assay. The cells exposed to MPP^+ at 1 mM with or without 100 μM Ami or 50 μM PF, and cells exposed to acidic medium with or without 100 μM Ami or 50 μM PF were used for LDH assay, flow cytometry, and Western blot analyses.

2.3. Lactate dehydrogenase (LDH) assay

To evaluate cell injury, the LDH released from cytosol into the culture medium was measured as described in a reference (Liu et al., 2005). LDH released from the cells exposed to the conditioned medium was determined using an assay kit according to the manufacturer's instructions (Nanjing Jiancheng Co., China). In brief, 100 μl cell-free supernatant, 250 μl buffer and 50 μl coenzyme were mixed and incubated for 15 min at 37 °C followed by addition of 250 μl 2,4-dinitrophenylhydrazine and another 15 min incubation at 37 °C in the dark. Finally, 2.5 ml NaOH (0.4 mol/l) was added to the reaction mixture. Three minutes later, 200 μl of each reaction mixture were transferred into the well of a new 96-well plate. The absorbance was read at 440 nm with an ELISA reader. Sample blank, standard, and standard blank tubes were measured simultaneous. The activity of LDH in a sample was calculated according to the formula:

$$\text{LDH activity(U/L)} = \left[\frac{\text{sample OD} - \text{sample blank OD}}{\text{standard OD} - \text{standard blank OD}}\right] \times 2\,\text{mmol/ml} \times 1000\,\text{ml}$$

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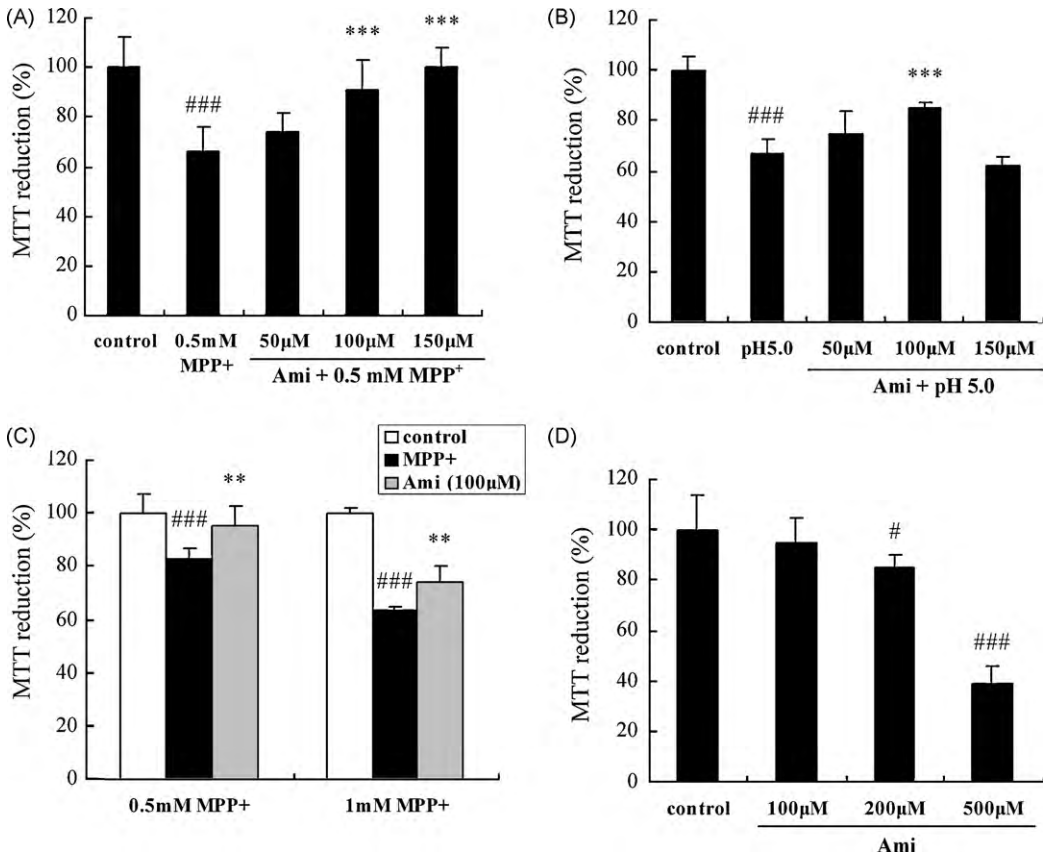

Fig. 1. MTT assays for the effects of Ami on PC12 cells. (A) The cell viability was significantly decreased after 24 h exposure to 0.5 mM MPP^+, however, Ami at a concentration of 100 and 150 μM blocked this effect significantly. (B) The cell viability was also decreased after 24 h exposure to acidic medium of pH 5.0, however, only 100 μM Ami blocked this effect. (C) Exposure PC12 cells to MPP^+ (0.5 and 1.0 mM) induced a dose dependent decrease of cell viability, while 100 μM Ami shown protective effect on both doses of MPP^+. (D) Ami at a concentration of 100 μM did not shown significant effect on PC12 cells treated with normal condition, but increased the concentration of Ami to 200 and 500 μM led to a dose dependence decrease on cell viability ($^{\#}p < 0.05$, $^{\#\#\#}p < 0.001$, vs. vehicle control group; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MPP^+/acidosis group).

2.4. Flow cytometric detection of apoptotic cells and cytosolic free Ca^{2+} concentrations

PC12 cells were harvested 24 h after exposure to the conditioned medium, washed twice with ice-cold Ca^{2+}-free PBS, and re-suspended in binding buffer at 1×10^6 cells/ml. Apoptotic rate was determined by flow cytometry using the Vybrant Apoptosis Assay Kit according to the manufacturer's instructions (Molecular Probes, USA). In brief, 100 μl of cell suspension was transferred into a tube and double-stained for 15 min with Alexa Fluor 488-conjugated annexin V (AV) and propidium iodide (PI) at room temperature in the dark. After addition of 400 μl binding buffer, the stained cells were analyzed with an FC500 flow cytometer using CXP cell quest software (Beckman-Coulter, USA). When measuring the fluorescence emission at 530 nm and >575 nm, "AV^-PI^-" represented normal cells; "AV^+PI^-", the early phase apoptotic cells; "AV^+PI^+", the late phase apoptotic cells; and "AV^-PI^+", the necrotic cells.

For cytosolic Ca^{2+} detection, 100 μl of cell suspension was incubated for 30 min at 37 °C with 10 μM fluo-3/AM (Sigma–Aldrich). After gently washing with ice-cold Ca^{2+}-free PBS, the stained cells were analyzed with the flow cytometer by detection of the fluorescence emission at 488 nm.

2.5. Western blot analyses for LC3 and LAMP2a proteins

Whole cell lysates were prepared from PC12 cells as described previously (Yang et al., 2009). Cells were collected after 24 h exposure to conditioned medium, washed three times with ice-cold PBS, and lysed by adding 50 μl lysis buffer (Beyotime Institute of Biotechnology, China), followed by a 10-min incubation on ice. The samples were sonicated for 16 s and then centrifuged for 15 min at 4 °C, 13,400 × *g*. After centrifugation, protein concentrations of the supernatants were ascertained using the BCA Protein Assay Kit (Beyotime). Samples containing 30 μg protein were mixed with sodium dodecyl sulfate (SDS)-Laemmli sample buffer (Beyotime) and boiled for 5 min to denature. Samples were fractionated on a SDS-PAGE gel (12% for LC3 and 8% for LAMP2a) and electrophoretically transferred onto PVDF membranes (Millipore, Bedford, MA). The membranes were blocked for 2 h with 5% non-fat milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 (TBST) at 37 °C, and then incubated overnight at 4 °C with polyclonal rabbit anti-LC3B antibody (1:1000, Abcam, MA), polyclonal rabbit anti-LAMP2a antibody (1:250, Abcam) or monoclonal mouse anti-beta-actin antibody (1:1000, Beyotime). After washing 3 × 10 min in TBST, the membranes were incubated with an HRP-conjugated secondary antibody (1:1000, Beyotime) for 2 h at 37 °C. After three washes in TBST, the protein was visualized with an ECL detection kit (Beyotime).

2.6. Statistics

Results are expressed as mean ± SD from at least three independent experiments. Two-group comparisons were performed using Student's *t*-test. Multiple-group comparisons were performed using one-way analysis of variance and Fisher's least significant difference (equal variances assumed) or Dunnett's T3-test (equal variances not assumed). Values of $p < 0.05$ were defined as statistically significant.

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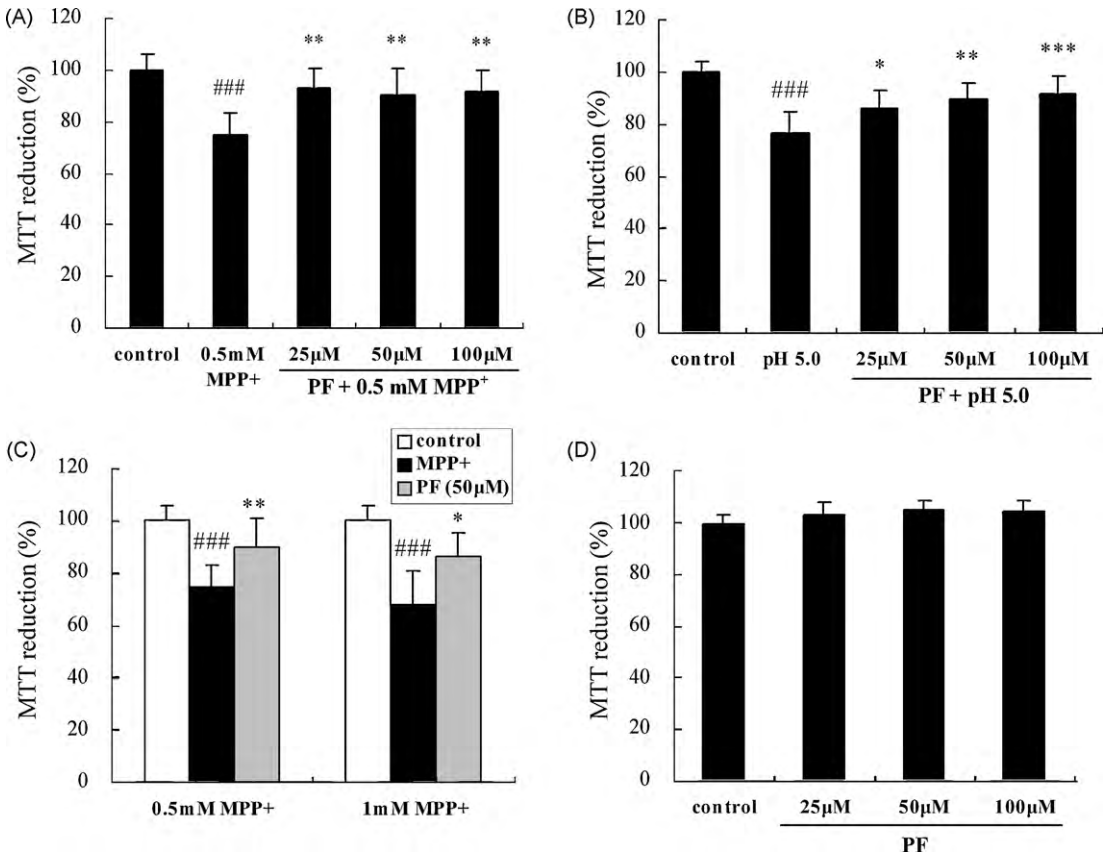

Fig. 2. MTT assays for the effects of PF on PC12 cells. (A) The cell viability was significantly decreased after 24 h exposure to 0.5 mM MPP^+, however, PF at a concentration of 25, 50 and 100 μM blocked this effect significantly. (B) The cell viability was also decreased after 24 h exposure to acidic medium of pH 5.0, however, PF at a concentration of 25, 50 and 100 μM blocked this effect. (C) Exposure PC12 cells to MPP^+ (0.5 and 1.0 mM) induced a dose dependent decrease of cell viability, while 50 μM PF shown protective effect on both doses of MPP^+. (D) PF at a concentration of 25, 50 and 100 μM did not shown significant effect on PC12 cells treated with normal condition ($^{###}p < 0.001$, vs. vehicle control group; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MPP^+/acidosis group).

3. Results

3.1. Ami attenuated MTT reduction induced by MPP^+/acidosis

Exposure of PC12 cells to 0.5 mM MPP^+ for 24 h significantly reduced cell viability. Addition of 100 or 150 μM of Ami restored viability almost to control levels, but 50 μM Ami had no effect (Fig. 1A). The acidic medium also markedly reduced viability. The addition of 100 μM Ami reduced the cytotoxicity significantly, 50 or 150 μM dide not (Fig. 1B). These results indicate that Ami at 100 μM attenuated the reduced viability induced by either 0.5 mM MPP^+ or acidosis.

To further determine the neuroprotective effects of Ami, we treated cells with different concentrations of MPP^+. Cell viability was significantly decreased following either 0.5 mM or 1.0 mM MPP^+, while addition of 100 μM Ami reversed their effects (Fig. 1C).

The potential toxicity of Ami to PC12 cells was examined by addition of Ami at 100, 200, and 500 μM to the vehicle control medium. Ami (100 μM) had no effect on the survival of PC12 cells. However, increasing the dose 200 μM and then 500 μM led to a progressive reduction of viability (Fig. 1D). These results suggest

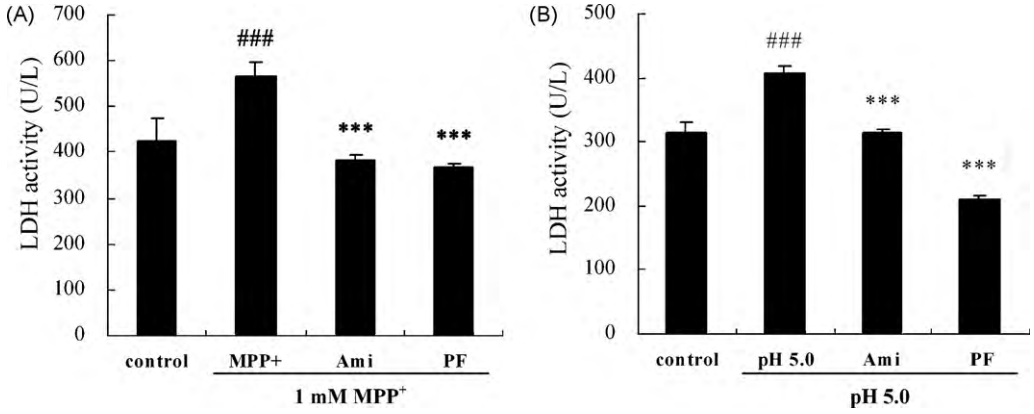

Fig. 3. LDH assay for the effects of Ami and PF on the PC12 cells exposed to MPP^+ (A) or acidic medium of pH 5.0 (B). $^{###}p < 0.001$ vs. vehicle control group; $^{***}p < 0.001$ vs. MPP^+/acidic group.

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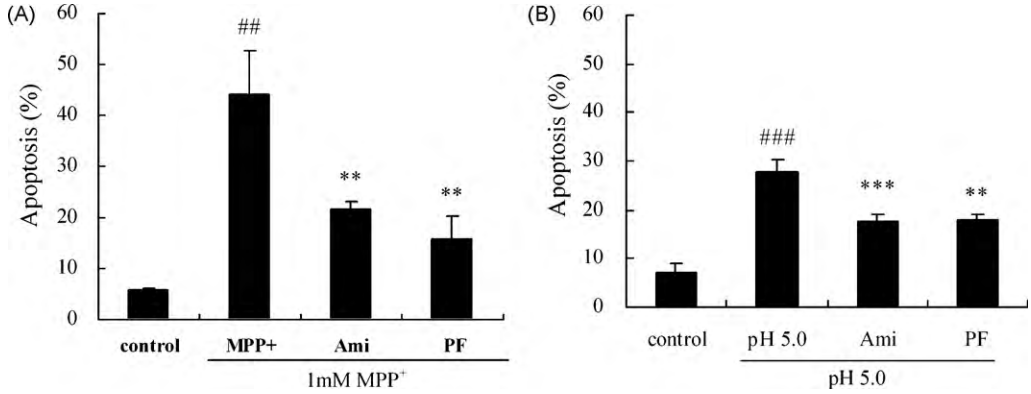

Fig. 4. Flow cytometry for the percentage of apoptosis cells. (A) Effects of Ami (100 μM) and PF (50 μM) on the cells treated with 1.0 mM MPP^+. (B) Effects of Ami (100 μM) and PF (50 μM) on the cells treated with acidic medium of pH 5.0 ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. vehicle control group; $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MPP^+/acidic group).

that 100 μM Ami is an effective and safe concentration to use with PC12 cells.

3.2. PF attenuated MTT reduction induced by MPP^+/acidosis

Treated cells with culture medium containing 0.5 mM MPP^+ significantly decreased cell viability, while addition of 25, 50, or 100 μM of PF restored viability almost to control levels (Fig. 2A). Addition of PF to acidic medium also restored viability (Fig. 2B).

We further determined the neuroprotective effects of 50 μM PF on the cells treated with 1.0 mM MPP^+, since there was not significant difference among the 25, 50, 100 μM of PF. Cell viability was significantly decreased following 1.0 mM MPP^+ treatment, while addition of 50 μM PF significantly increased viability (Fig. 2C).

We also demonstrated that PF at doses of 25, 50, and 100 μM did not have significant effects on the promotion of PC12 cell proliferation when the cells were cultured in vehicle control medium (Fig. 2D). Thus, it is possible to conclude that PF is effective in the neuroprotection of PC12 cells.

3.3. Ami and PF inhibited LDH release from cells treated with MPP^+/acidosis

Lactate dehydragenase (LDH) is a stable enzyme, present in all cell types, and rapidly released into the medium upon damage of the plasma membrane. A 24-h exposure to 1.0 mM MPP^+ resulted in marked facilitation of LDH release from the cells, significantly different from the vehicle control group. In contrast, the LDH activity of supernatant was significantly decreased in the cells treated with 100 μM Ami or 50 μM PF plus 1.0 mM MPP^+ (Fig. 3A).

Results were very similar for the cells exposed to acidic medium. Acidic application for 24 h led to an increase of supernatant LDH, significantly greater than vehicle control group, while the addition of 100 μM Ami or 50 μM PF both effectively inhibited acid-induced LDH release (Fig. 3B).

These data suggested that Ami and PF offered almost complete protection against both MPP^+ and acid-induced cell death, and the viability was restored nearly to the control level. Taken together, these results allow us to conclude that Ami and PF both were effective for the protection of PC12 cells.

3.4. Ami and PF reduced apoptotic rate in cells treated with MPP^+/acidosis

Double staining of treated PC12 cells with annexin V (marker for apoptosis) and PI (indicator of necrotic cells) was assayed with flow cytometry to further determine the apoptotic rate in each group. After 24 h treatment with 1.0 mM MPP^+ alone, the percentage of apoptotic cells increased in comparison with the vehicle control, but dropped significantly when treated with 100 μM Ami or 50 μM PF plus MPP^+ (Fig. 4A).

Similarly, exposure to acidic medium for 24 h revealed that the percentage of annexin V-positive cells was significantly higher than in the vehicle control group. Simultaneous incubation with 100 μM Ami or 50 μM PF significantly reduced the number of cells labeled

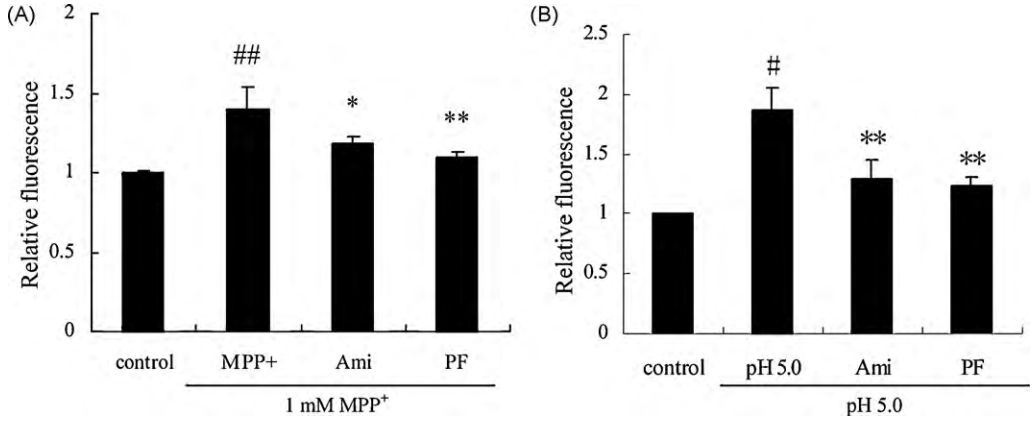

Fig. 5. Flow cytometry for the fluorescence intensity of fluo-3/AM. (A) Effects of Ami (100 μM) and PF (50 μM) on the cells treated with 1.0 mM MPP^+. (B) Effects of Ami (100 μM) and PF (50 μM) on the cells treated with acidic medium of pH 5.0 ($^{\#}p < 0.05$, $^{\#\#}p < 0.01$, vs. vehicle control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. MPP^+/acidic group).

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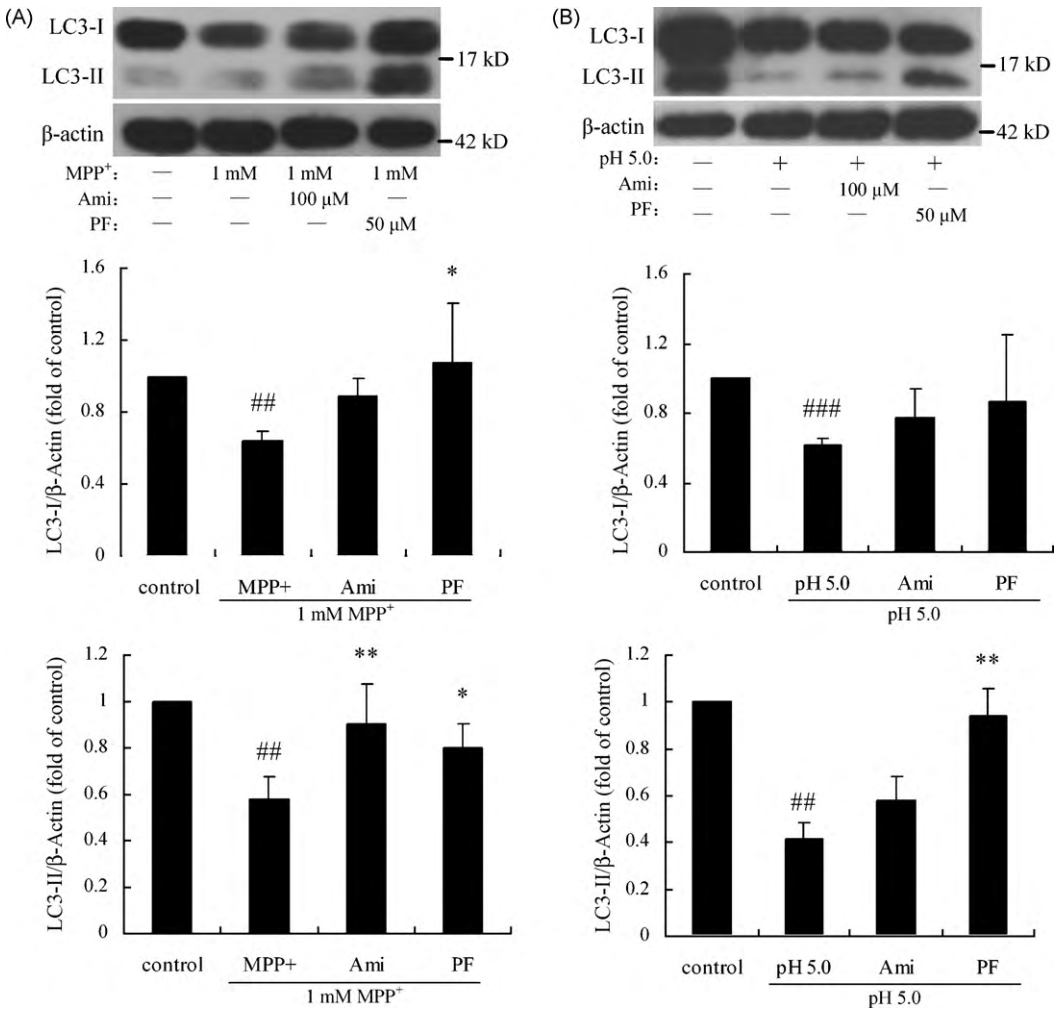

Fig. 6. Western blot analysis for LC3 protein. (A) The LC3-I and LC3-II levels are both reduced after 24 h exposure to 1.0 mM MPP^+ and this decrease effects are blocked when cell co-incubated with 100 μM Ami or 50 μM PF. (B) The LC3-I and LC3-II levels of cells exposed to acidic medium are also reduced, and again the effects are blocked in the presence of Ami or PF. β-Actin protein was used in these experiments as the loading control ($^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$, vs. vehicle control group; $^{*}p < 0.05$, $^{**}p < 0.01$, vs. MPP^+/acidic group).

with annexin V and the percentage of apoptotic cells decreased markedly (Fig. 4B).

3.5. Ami and PF reduced cytosolic free Ca^{2+} concentrations

An overload of intracellular calcium ions is widely considered to be one of the molecular mechanisms of neuronal death. Fluo-3/AM has been used to determine the concentrations of cytosolic free Ca^{2+} by measuring the fluorescence intensity. Exposure of PC12 cells to 1.0 mM MPP^+ significantly increased the fluorescence intensity relative to the vehicle control group. However, addition of 100 μM Ami or 50 μM PF significantly inhibited the calcium influx (Fig. 5A). As expected, acid application also markedly increased the fluorescence intensity, but simultaneous incubation with 100 μM Ami or 50 μM PF effectively reduced it (Fig. 5B).

3.6. Ami and PF upregulated the expression of LC3-I and LC3-II protein

We found that the expression of LC3-I (the cytosolic precursor of LC3-II) and LC3-II (a specific marker of phagophores and autophagosomes) both decreased when cells were exposed to 1.0 mM MPP^+ for 24 h relative to the vehicle control group. Addition of 100 μM Ami increased both LC3-I and LC3-II, but only the latter showed significant change. Addition of 50 μM PF sharply increased LC3-I and LC3-II (Fig. 6A).

When PC12 cells were exposed to acidic medium for 24 h, the protein levels of LC3-I and LC3-II decreased relative to the vehicle control group. Although Ami and PF both increased the protein levels of LC3-I, a one-way ANOVA showed that the difference was not statistically significant ($F = 0.832$; 2, 6 d.f., $P = 0.480$). However, PF significantly increased the levels of LC3-II (Fig. 6B).

3.7. Ami and PF inhibited the overexpression of LAMP2a protein induced by MPP^+/acidosis

CMA is a selective pathway for the degradation of cytosolic proteins in lysosomes. It has been reported that CMA is activated during oxidative stress (Kaushik and Cuervo, 2006). Since autophagy is inhibited by either MPP^+ or acidosis, we then analyzed whether they altered CMA activity. Interestingly, we found that the expression level of LAMP2a was significantly increased in the cells treated with MPP^+ alone compared with vehicle control cells. However, 100 μM Ami or 50 μM PF effectively reversed the induction of CMA, as reflected in a reduction of the expression of LAMP2a (Fig. 7A).

We obtained the similar results on cells treated with acid. Acid application significantly increased the level of LAMP2a compared

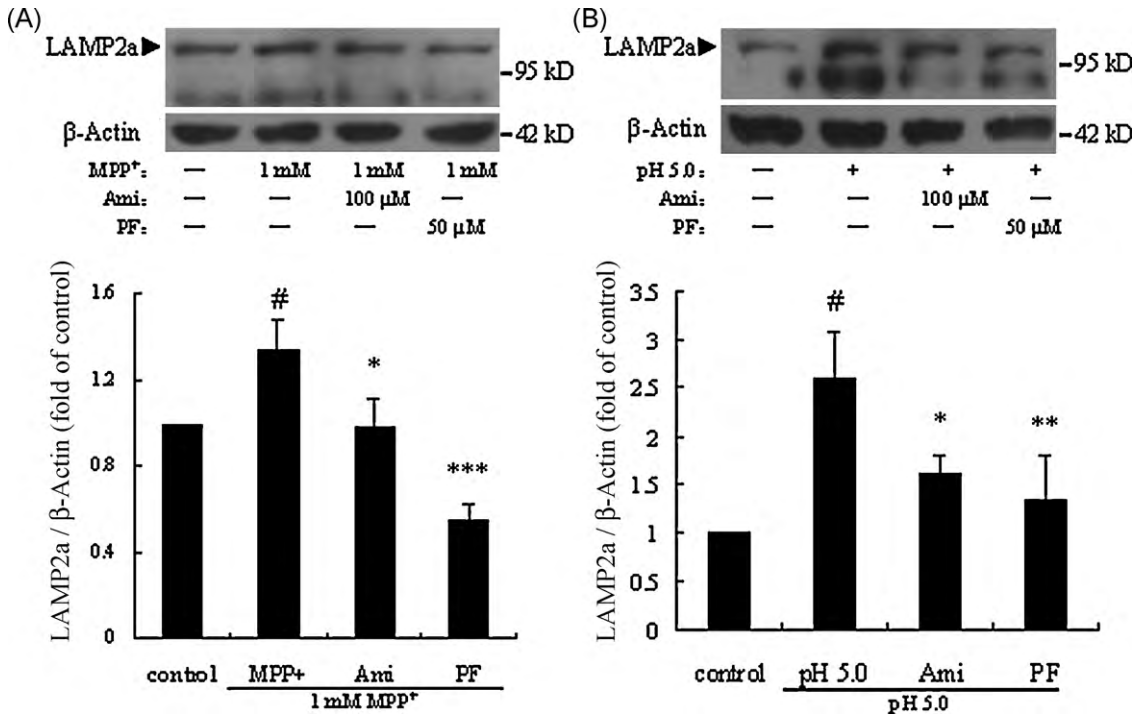

Fig. 7. Western blot analysis for LAMP2a protein. (A) The LAMP2a level is increased after 24 h exposure to 1.0 mM MPP^+ and this increase is blocked when cell co-incubated with 100 μM Ami or 50 μM PF. (B) The LAMP2a level of cells exposed to acidic medium is also increased, and again the effect is blocked in the presence of Ami or PF. β-Actin protein was used in these experiments as the loading control ($^{\#}p < 0.01$ vs. vehicle control group; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, vs. MPP^+/acidic group).

with control cells, while 100 μM Ami and 50 μM PF both effectively decreased LAMP2a (Fig. 7B).

4. Discussion

In this study, we demonstrated that PF is an effective compound in the modulation of autophagic activity. Treatment with MPP^+ significantly inhibited autophagy and induced apoptosis in PC12 cells. However, co-treatment with PF (50 μM) upregulated the autophagy and clearly showed neuroprotective effects. In addition, the CMA pathway was activated as a compensatory mechanism (Kaushik et al., 2008), while PF efficiently maintained the CMA activity near the normal range. We also exposed PC12 cells to an acidic medium, since acidosis is a common cause of mitochondrial dysfunction and neuron degeneration. Similarly, the deficiency of autophagy and the overactivity of CMA were both attenuated by PF. Thus, the key finding of the present study was that PF potently protected PC12 cells against MPP^+ or acidosis-induced injury by upregulating the autophagic pathway.

In addition, several lines of evidence have converged to suggest that ASICs, which have been found to underlie calcium transients, contribute to neuronal cell death associated with extracellular acidosis (Yermolaieva et al., 2004), in conditions such as seizures (Ziemann et al., 2008), cerebral ischemia (Liu et al., 2009), neuroinflammation (Friese et al., 2007), and PD (Arias et al., 2008). ASICs are H^+-gated cation channels, which belong to the degenerin/epithelial sodium channel superfamily (Jasti et al., 2007). They are activated quickly as the extracellular pH falls. At the same time, ASICs show some permeability to Ca^{2+} and the relative Ca^{2+} permeability is highest in ASIC1a homomers and ASIC1a/ASIC2a heteromers, which are highly expressed in the brain (Wu et al., 2004; Wong et al., 2008). A growing body of evidence has identified ASIC1a as a possible therapeutic target for the reduction of calcium influx (Wang et al., 2006). Amiloride, a licensed and clinically safe blocker of ASICs, has been shown to be neuroprotective in rodent models of PD (Arias et al., 2008), Huntington's disease (Wong et al., 2008), multiple sclerosis (Friese et al., 2007), and traumatic brain injury (Turner et al., 2004). It is therefore reasonable to assume that Ami has neuroprotective effects on PC12 cells. Thus, we choose Ami as a positive control drug and found that it effectively protected PC12 cells against MPP^+/acidic injury. From our studies, Ami at a concentration of 100 μM increased the viability of cells exposed to MPP^+ (0.5 and 1.0 mM) or acidic medium and without significant effect on cells cultured under normal conditions. Ami decreased the release of LDH from PC12 cells as well as the percentage of apoptotic cells. Though we do not have direct experimental data showing that ASIC1a is indeed activated during pH fluctuations or MPP^+ exposure, we have provided unambiguous evidence that Ami has a beneficial effect in cellular models of PD.

Furthermore, we showed that the $(Ca^{2+})_i$ in cells exposed either to acidic medium or to MPP^+ increased in comparison with control cells, and Ami decreased the intracellular calcium concentration. Similarly, PF also diminished calcium influx in cells exposed either to acidic medium or to MPP^+. Although some studies indicated that the neuroprotective effect of PF is closely associated with its activity in maintenaning $(Ca^{2+})_i$ (Zhong et al., 2009), little is known about the precise mechanism. It has been reported that the pH for half-maximal activation (pH_{50}) of ASIC1a, ASIC2a, ASIC1a/2a is ~6.2, 4.1, ~4.8 respectively (Chu et al., 2002; Wu et al., 2004). Therefore, exposing PC12 cell to an acidic medium of pH 5.0 may activate ASICs followed by calcium influx. Our data support the notion that PF may reduce calcium influx by inhibiting ASICs, however, further research is needed to explain the precise mechanism(s) in detail.

An interesting discovery in this study is that autophagic activity is also elevated by the inhibition of ASICs. Ami significantly attenuated the inhibition of autophagy and downregulated the overactivition of CMA. To the best of our knowledge, a direct connection between ASICs and ALP activity has not been established, although some studies suggest that ASICs are linked to the

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ubiquitin-proteasome system (UPS), another protein degradation pathway. Wong et al. (2008) have reported that the Ami derivative benzamil alleviates the inhibition of UPS activity, and importantly, blocks the expression of ASIC1a, leading to an enhancement of UPS activity. Furthermore, mutations in the parkin gene result in an autosomal recessive juvenile-onset form of PD. Joch et al. (2007) have reported that defects in Parkin-mediated PICK1 monoubiquitination enhance ASIC activity and thereby promote neurodegeneration in PD. Another study has demonstrated that ASIC4 interacts strongly with polyubiquitin (Donier et al., 2008). Taken together, these data suggest that ASICs indeed alter the function of protein degradation pathways. Our preliminary findings represent the first step in understanding the relationships between ASICs and ALP.

In summary, the main findings of this study are: (1) PF and Ami both potently protected PC12 cells against MPP^+ or acidosis-induced injury by upregulating the autophagic pathway; (2) PF reduced calcium influx by inhibiting ASIC1a channels; (3) the activity of ASICs altered the function of ALP.

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