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GPR30 Activation Contributes to the Puerarin-Mediated Neuroprotection in MPP$^+$-Induced SH-SY5Y Cell Death

Yue-Fa Cheng[1] · Guoqi Zhu[2] · Qing-Wen Wu[3] · Yue-Sheng Xie[1] · Yan Jiang[1] · Lan Guo[1] · Ya-Li Guan[1] · Ying-Shuo Liu[1] · Jun Zhang[1]

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Abstract The neuroprotective action of puerarin in Parkinson's disease (PD) models has been well investigated. However, the mechanisms involved in protection have not been completely understood. G protein-coupled receptor 30 (GPR30) is a G protein-coupled estrogen receptor and considered a potential target in the neuroprotection against PD. In this study, we investigated whether puerarin prevented against 1-methyl-4-phenylpyridinium (MPP$^+$)-induced cell death via GPR30. Our results showed that the GPR30 agonist, G1, exhibited puerarin-mediated neuroprotection against MPP$^+$-induced cell death of SH-SY5Y cells. This protective action was reversed by the GPR30 antagonist. Moreover, a time- and concentration-dependent effect of puerarin on GPR30 expression was verified at the protein level but not at the mRNA level. Further, we showed that an mTor-dependent new GPR30 synthesis contributed to the protection conferred by puerarin. Finally, glial cell line-derived neurotrophic factor (GDNF) levels were enhanced by puerarin and G1 in both control and MPP$^+$-lesioned cells via GPR30. Taken together, our data strongly suggest that puerarin prevents MPP$^+$-induced cell death via facilitating GPR30 expression and GDNF release.

Yue-Fa Cheng and Guoqi Zhu contributed equally to this work.

✉ Yue-Fa Cheng
chengyuefa@hotmail.com

[1] Jitang College of North China University of Science and Technology, Tangshan 063000, China

[2] Key Laboratory of Xin'an Medicine, Ministry of Education, Anhui University of Chinese Medicine, Hefei 230038, China

[3] College of Nursing and Rehabilitation, North China University of Science and Technology, Tangshan 063000, China

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Introduction

Parkinson's disease (PD) is a severe neurodegenerative disease symptomized by both motor and non-motor dysfunctions. Dopaminergic neuronal death was considered the major pathological cause for these deficits (Surmeier et al. 2010). Mitochondrial membrane impairment, radical oxidative stress, and dysfunction of protein degradation systems are considered the three major causative factors for dopaminergic cell death (Malkus et al. 2009). Compounds with the potential to reverse the impairment of mitochondrial membrane potential, antagonize oxidative stress, or promote the degradation of accumulated proteins are effective in the prevention of neuronal death (Cheng et al. 2009; Javed et al. 2016; Ojha et al. 2016).

G protein-coupled receptor 30 (GPR30) is a G protein-coupled estrogen receptor mainly located on the cell membrane. GPR30 is different from the traditional estrogen receptors for its location and mechanism of action (Jacenik et al. 2016). The classical estrogen receptors (ERα and ERβ) mainly function through their genomic responses to 17β-estradiol (E2), whereas GPR30 activation is mediated by non-genomic responses (Tran et al. 2016). E2 is reportedly neuroprotective as per various experimental and clinical studies (Al Sweidi et al. 2012; Lebesgue et al. 2009). However, long-term clinical utility of E2 is hampered by elevated risk for breast and uterine cancers owing to its genomic nuclear response. G1, a GPR30 agonist, shows high selectivity in GPR30 activation, thus avoiding the genomic response and adverse effects. E2 along with some phytoestrogens are also known to activate GPR30 to exert their biological activities (Kim et al. 2016; Lee et al. 2014). Interestingly, GPR30 shows neuroprotective functions

(Tang et al. 2014) as well as contributing to hippocampal synaptic plasticity (Briz et al. 2015a). In PD, GPR30 activation also prevented dopaminergic cell death likely through the activation of striatal Akt signaling and increase in Bcl-2 and brain-derived neurotrophic factor (BDNF) levels (Bourque et al. 2013; Bourque et al. 2015; Cote et al. 2015).

A variety of components extracted from Chinese herbs have neuroprotective action in PD (Cheng et al. 2009; Zhao et al. 2016). Puerarin ($C_{21}H_{20}O_9$) is one such active component, which was purified from *Pueraria lobata* (Willd.) and Ohwi *Pueraria thomsonii* Benth. Puerarin-mediated neuroprotection has been well documented both in in vitro and in vivo PD models (Cheng et al. 2009; Zhu et al. 2010; Zou et al. 2013) likely through apoptosis inhibition and release of anti-oxidant and neurotropic factors (Zhu et al. 2014). However, the exact target involved in this mechanism is not well understood. With a chemical structure of isoflavone, puerarin also exhibits neuroprotection via its weak estrogen-like activity (Ji et al. 2013; Zou et al. 2013). Glial cell line-derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many neuron types, especially dopaminergic neurons. We previously reported that puerarin facilitated GDNF expression in a 6-OHDA-induced PD model (Zhu et al. 2012). In this study, we further investigate the exact target involved in puerarin-mediated neuroprotection in 1-methyl-4-phenylpyridinium (MPP^+)-induced cell death and GDNF decrease.

Materials and Methods

Reagents

The companies from which antibodies were purchased are listed as follows: anti-phospho-mTor (Ser-2448, Santa Cruz, USA), anti-mTor (Santa Cruz, USA), anti-phospho-p70S6 K (CST, USA), anti-p70S6 K (CST, USA), anti-GPR30 (Novus Biologicals, USA), and anti-actin (CST, USA). Puerarin, MPP^+, and 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA).

Groups and Drug Treatment

SH-SY5Y cell line (ATCC, USA) was cultured in an incubator with 5 % CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (Hyclone, Logan, UT), 100 U/ml penicillin, and 100 mg/ml streptomycin. The following study groups were included: control, 1 mM MPP^+, 1–100 μM puerarin + MPP^+, G1 + MPP^+, GPR30 antagonist (G15) + 50 μM puerarin + MPP^+, G1, G15, and puerarin groups. The cells were incubated with puerarin for 3 h before MPP^+ insult; cells in the G15 group underwent pre-treatment with G15 prior to puerarin treatment. Puerarin, G1, and G15 were dissolved in dimethyl sulfoxide (DMSO; final concentration less than 0.1 %). All the experimental protocols were approved by the ethics committee of North China University of Science and Technology.

Cell Viability

SH-SY5Y cells in logarithmic growth phase were seeded in 96-well culture plates. After treatment with the indicated drugs for 48 h, 20 μl MTT (5 mg/ml) was added to the 200-μl culture medium of each well. Four hours later, the medium was removed, and 150-μl DMSO was added into each well in order to dissolve the precipitation. The absorbance (*A*) was measured at a wavelength of 490 nm using an automated microplate reader (Multiskan FC, Thermo Scientific). Cell viability was calculated by the following formula: cell viability (%) = average absorbance of treated group/average absorbance of control group × 100 %.

Total RNA Extraction and Semi-Quantitative PCR

After treatment with the indicated drugs, the cells were collected and total RNA was isolated using TRIzol Reagent according to the manufacturer's instructions (Thermo Fisher Scientific, USA). Briefly, 2 μg RNA was reverse-transcripted using the reverse-transcription system (Promega Corporation, USA). The following primers (synthesized by Shenggong, Shanghai) were employed for GPR30 amplification:

Forward 5′-GACGACCTCAACGCACAGTA-3′;
Reverse 5′-AGGAGTCCCATGATGAGATTGT-3′.

Actin primers were as follows:

Forward 5′-AAGGACTCCTATAGTGGGTGACGA-3′
Reverse 5′-ATCTTCTCCATGTCGTCCCAGTTG-3′.

PCR amplification was carried out under the following conditions: an initial holding at 95 °C for 10 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 45 s.

Immunoblot Analysis

The extraction of cytoplasmic protein was performed according to the Cytoplasmic Protein Extraction Kit (Beyotime, Jiangsu, China). After treatment with the indicated drugs, the cells were washed with 1-ml ice-cold PBS, collected, and centrifuged for 5 min at 1200 rpm at 4 °C. The pellet was dissolved with cytoplasmic protein extraction agent A supplemented with 1 mM PMSF. After a 5-s vortex, the tubes

were incubated for 10–15 min on ice to promote cell lysis. Then, the cytoplasmic protein extraction agent B was added. The samples were then centrifuged for 5 min at 14,000*g* at 4 °C, and the supernatant was collected for further analysis.

Protein concentration was quantified by the BCA method (Beyotime, Jiangsu, China), and an equal amount of protein (20 μg) was loaded onto 10 % sodium dodecyl sulfate-polyacrylamide gels for electrophoresis (SDS-PAGE). After electrophoresis, proteins were transferred onto a nitrocellulose membrane. Non-specific protein binding was blocked by 4 % defat milk. The membranes were incubated with anti-phospho-mTor (Ser-2448, 1:1000), anti-mTor (1:1000), anti-phospho-S6 K (1:1000), anti-S6 K (1:1000), anti-GPR30 (1:1000), and anti-actin (1:5000) at 4 °C overnight. After washing, the membranes were incubated with a secondary antibody (peroxidase-labeled anti-mouse antibody; 1:5000 dilution). The signal was detected using an enhanced chemiluminescence detection kit (Amersham ECL RPN 2106 Kit, Amersham Pharmacia Biotech, QC, Canada).

De novo protein synthesis was determined by using metabolic labeling with Click-iT L-azidohomoalanine (AHA, Molecular Probes) as previous described, with minor modification (Wang et al. 2014). The cells were pre-incubated with 500 mM AHA in the absence and presence of 1 μM rapamycin for 20 min and then treated with 50 μM puerarin for 3 h. After rinsing with PBS, the protein was isolated and samples were incubated with phosphine-PEG3-Biotin (Thermo) for 4 h at 37 °C to conjugate biotin to AHA-containing proteins. Samples were then eluted on Zeba Spin desalting columns (7 K MWCO; Thermo) to remove the excess of phosphine-PEG3-biotin. Equal amounts of proteins were incubated with GPR30 antibody (1:100) overnight at 4 °C. Subsequently, 50-ml protein A-sepharose beads (1:1, Sigma) were added to each sample and incubated for 1 h at 4 °C with gentle rocking. After three washes in PBS, samples were processed for SDS-PAGE and western blotting. IRDye 800CW streptavidin (1:2000, LI-COR Biosciences) was used to detect biotin-conjugated (newly synthesized) GPR30. Anti-GPR30 antibody (1:1000; Novus Biologicals, USA) was used to detect total immunoprecipitated GPR30.

Immunochemical Staining

After treatment with indicated drugs, the cells were fixed in 4 % paraformaldehyde. Non-specific staining was blocked by 0.1 M PBS containing 10 % goat serum with 0.4 % Triton X-100. The cells were mounted on a coverslip and incubated with anti-GPR30 antibody (1:400) in 0.1 M PBS containing 5 % goat serum and 0.4 % Triton X-100 overnight at 4 °C. Sections were washed three times (15 min each) in PBS and incubated with Alexa Fluor 594 goat anti-rabbit IgG (Life Technologies, USA) for 2 h at room temperature. Fluorescent images were captured using the Olympus fluorescence microscopy (Olympus, Japan).

GDNF Assay

SH-SY5Y cells in logarithmic growth phase were seeded in a 48-well culture plate. After treatment with the indicated drugs for 48 h, a 100-μl aliquot of the supernatant was analyzed using enzyme-linked immunosorbant assay (Rat GDNF PicoKine™ ELISA Kit, Boster, CA, USA). The absorbance was determined in a microplate reader at 450-nm wavelength (Multiskan FC, Thermo Scientific).

Statistical Analysis

The values are presented as means ± standard error of mean (SEM). Statistical analyses of the data were performed using Student's *t* test, one-way analysis of variance, and Newman–Keuls multiple comparisons. $P < 0.05$ was considered statistically significant for all analyses.

Results

Puerarin Prevented MPP^+-Induced Cell Death via Activating GPR30

After treatment with 1 mM MPP^+ for 48 h, the cell viability was about 60 %. Puerarin at concentrations of 50 and 100 μM significantly decreased MPP^+-induced cell loss. Interestingly, we found that the effect of 50 μM puerarin was blocked by G15 (1 μM) (Fig. 1a). Moreover, pre-treatment with the GPR30 agonist G1 (2 μM) also decreased MPP^+-induced cell death. No difference in cell viability was observed in the control, 50-μM puerarin, G1, or G15 alone groups (Fig. 1b). These data showed that puerarin moderated MPP^+-induced cell death via GPR30 activation.

Puerarin Activated mTor Signaling Pathway to Increase GPR30 Expression

As shown in Fig. 2a, puerarin promoted GPR30 expression in SH-SY5Y cells in a concentration-dependent manner. Puerarin (10 μM) slightly increased GPR30 expression (1.2-fold) after 24-h treatment, while it enhanced GPR30 expression to peak level (1.5-fold) at 50 μM. To verify the time-dependent effect of puerarin on GPR30 expression, we selected a concentration of 50 μM puerarin in the experiment. As shown in Fig. 2b, GPR30 expression was enhanced 3 h after 50-μM puerarin treatment, and the effects lasted for at least 24 h. By contrast, GPR30 messenger RNA (mRNA) level was not influenced by puerarin application (Fig. 2c, d). In addition,

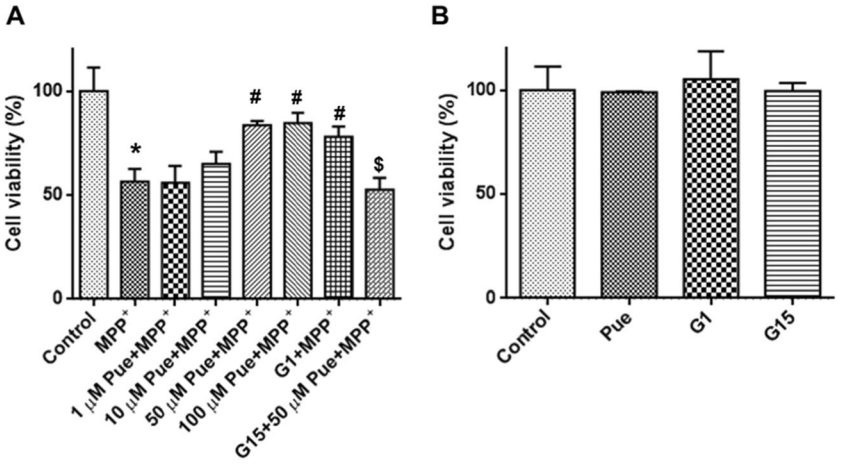

Fig. 1 Puerarin prevents against MPP^+-induced SH-SY5Y cell death via activating GPR30. **a** Puerarin (1–100 μM) prevents against MPP^+-induced SH-SY5Y cell death in a concentration-dependent manner. G1 prevents against MPP^+-induced SH-SY5Y cell death, while G15 blocks the effects of puerarin on MPP^+-induced SH-SY5Y cell death. **b** Puerarin (50 μM), G1, or G15 alone did not affect the cell viability. Data are presented as mean ± SEM with six repeats. $^*p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with MPP^+, $^{\$}p < 0.05$ compared with 50 μM puerarin + MPP^+. *Pue* puerarin, *G1* GPR30 agonist, *G15* GPR30 antagonist

immunofluorescent staining was also used to detect GPR30 expression after puerarin treatment. As shown in Fig. 2e, GPR30 was extensively expressed in the cell membrane or cytoplasm but not in the nucleus. After exposure of the cells in puerarin, GPR30 was markedly up-regulated at both the 3- and 6-h time points.

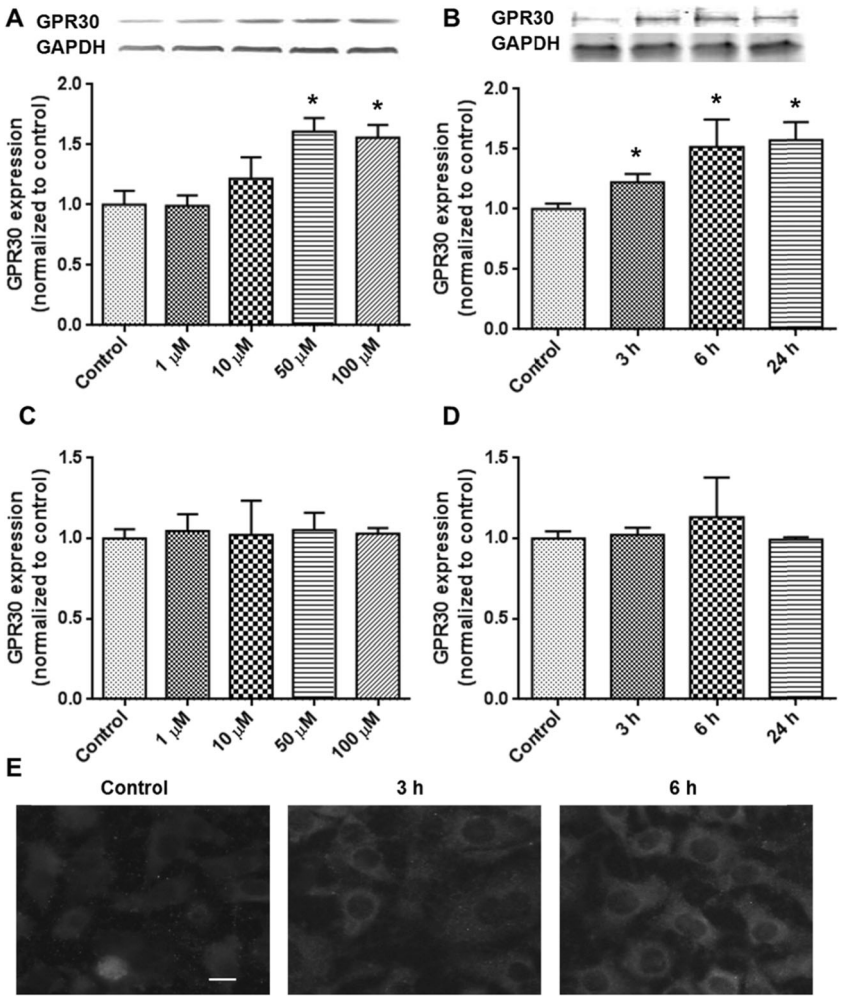

Fig. 2 Puerarin up-regulates GPR30 protein expression. **a** Puerarin increases the protein levels of GPR30 in a concentration-dependent manner (24-h treatment). **b** Puerarin (50 μM) increases GPR30 expression in a time-dependent manner. **c** Different concentrations of puerarin (1–100 μM) did not affect GPR30 expression detected by real-time PCR (24-h treatment). **d** Treatment with puerarin (50 μM) for different times (3–24 h) did not affect GPR30 expression detected by real-time PCR. **e** Immunofluorescence staining detecting GPR30 expression 3 and 6 h after puerarin treatment. *Scale bar* 10 μm. Data are presented as mean ± SEM with six repeats. $^*p < 0.05$ compared with control

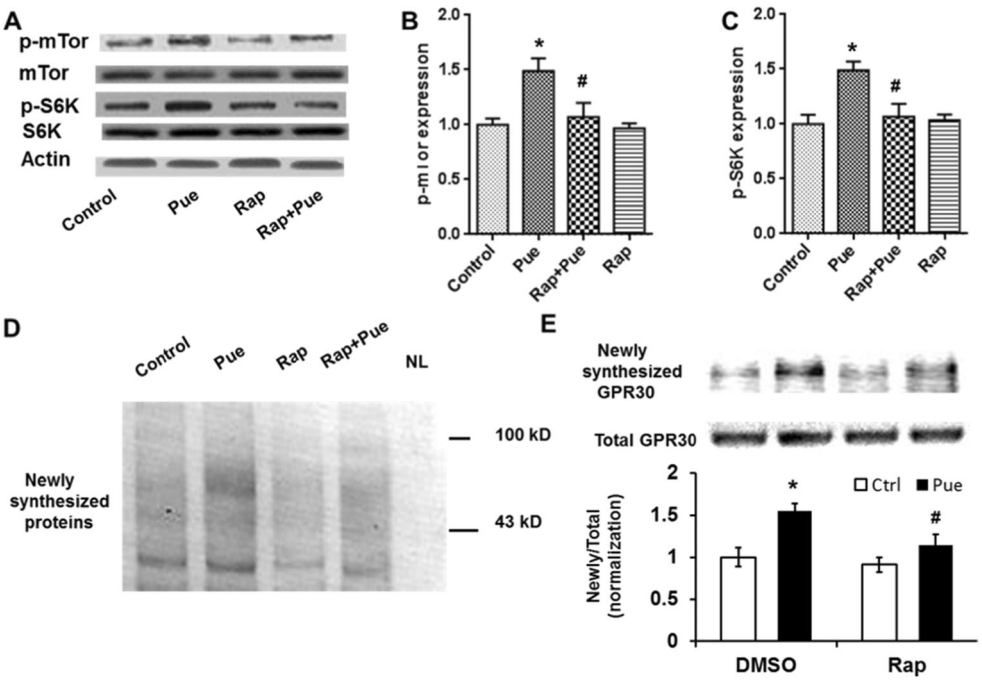

Fig. 3 Puerarin up-regulates GPR30 expression via activation of the mTor signaling pathway. **a** Puerarin treatment (50 μM) activates the p-mTor pathway. Representative blots of p-mTor, p-p70S6K, mTor, and p70S6K. **b** Quantification data of p-mTor/mTor. **c** Quantification data of p-p70S6K/p70S6K. **d** Puerarin treatment promotes newly synthesized proteins. **e** Puerarin treatment promotes newly synthesized GPR30. *upper panel* Representative blots and *lower panel* quantification data. Data are presented as mean ± SEM with six repeats. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with corresponding DMSO. *Pue* puerarin, *Rap* rapamycin, *NL* non-labeling

The signaling pathway involved in the puerarin effect was also accessed. As shown in Fig. 3a, b, p-mTor was significantly activated 3 h after puerarin treatment. The downstream effector of mTor, p70S6K, was also significantly phosphorylated. Rapamycin pre-treatment attenuated the phosphorylation of mTor and p70S6K. By contrast, puerarin or rapamycin alone did not affect the total mTor and p70S6 K levels.

In order to examine whether puerarin promoted de novo GPR30 synthesis, we performed experiments in SH-SY5Y cells using a modified amino acid, L-azidohomoalanine, which is incorporated into newly synthesized proteins and can later be labeled with biotin. Total GPR30 was immunoprecipitated after metabolic labeling of nascent proteins. Newly synthesized (biotin labeled) and total GPR30 were then detected using infrared dye-conjugated streptavidin and anti-GPR30 antibody, respectively. Treatment of the cells with puerarin for 3 h significantly increased the levels of newly synthesized GPR30 as compared with controls (Fig. 3c). Rapamycin also blocked the newly synthesized GPR30 (Fig. 3d). These results further supported that puerarin incubation stimulated GPR30 protein expression, possibly through activating the mTor signaling pathway.

We also detected the effect of puerarin on GPR30 expression in MPP^{+}-lesioned cells. As shown in Fig. 4a, MPP^{+} did

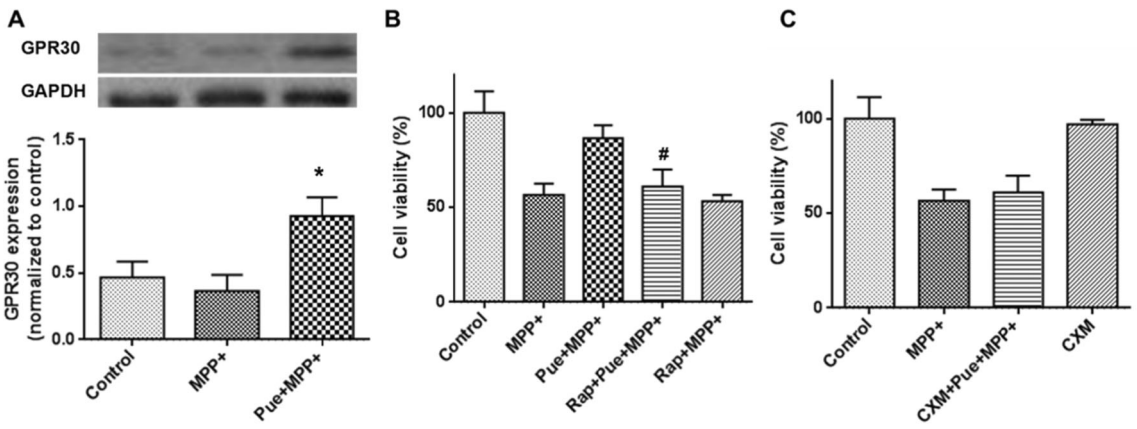

Fig. 4 Puerarin prevents against MPP^{+}-induced cell death via promoting GPR30 expression. **a** Puerarin promotes GPR30 expression in MPP^{+}-induced cells. *upper panel* Representative blots and *lower panel* quantification data. **b** Rapamycin blocks puerarin-mediated protection of MPP^{+}-induced cell death. **c** Cycloheximide blocks puerarin-mediated protection of MPP^{+}-induced cell death. $^{*}p < 0.05$ compared with MPP^{+}, $^{\#}p < 0.05$ compared with puerarin + MPP^{+}. *Pue* puerarin, *Rap* rapamycin, *CXM* cycloheximide

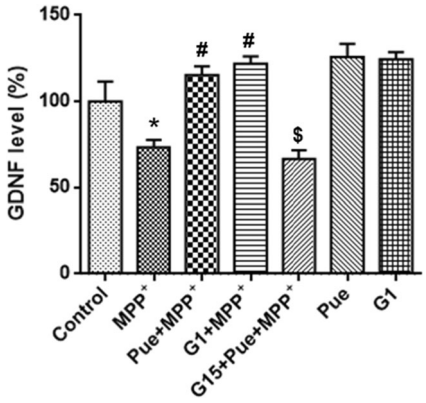

Fig. 5 GPR30 activation by puerarin stimulates GDNF release. GPR30 agonist has similar effect as puerarin, while GPR30 antagonist blocks the effect of puerarin. G1 or puerarin alone does not affect GDNF in SH-SY5Y cells. $^{*}p < 0.05$ compared with control, $^{\#}p < 0.05$ compared with MPP^{+}, $^{\$}p < 0.05$ compared with MPP^{+}. *Pue* puerarin, *G1* GPR30 agonist, *G15* GPR30 antagonist

not affect GPR30 expression, while puerarin remarkably increased GPR30 in MPP^{+}-treated cells. As rapamycin blocked the new synthesis of GPR30, it should ideally also block the effect of puerarin on MPP^{+}-induced cell death. As confirmed by MTT assay, puerarin protection was blocked by rapamycin pre-treatment (Fig. 4b). In addition, a protein synthesis inhibitor (cycloheximide, 1 μM) was also found to block the puerarin-mediated protection (Fig. 4c). These data suggested that the new protein synthesis of GPR30 was involved in puerarin-mediated protection of MPP^{+}-induced cell death.

GPR30 Activation Prevented MPP^{+}-Induced Decrease of GDNF

GDNF levels were detected after the indicated drug treatments in SH-SY5Y cells. We found that incubation with MPP^{+} decreased the GDNF level. Puerarin or GPR30 agonist activated GDNF level not only in normal but also in MPP^{+}-induced SH-SY5Y cells (Fig. 5). Moreover, G15 blocked the effect of puerarin on GDNF expression in SH-SY5Y cells that underwent MPP^{+} treatment.

Discussion

In this study, we report the novel action of puerarin via GPR30 expression in MPP^{+}-treated SH-SY5Y cells. GPR30 was up-regulated at the protein level, but not in the mRNA level, and this up-regulation of GPR30 played a critical role in preventing MPP^{+}-induced cell death. We believe that GPR30 could be a potential target for PD therapy, consistent with previous studies (Bourque et al. 2013; Tang et al. 2014).

Similarly, consistent with our previous study, others have shown that puerarin prevented against neurotoxin-induced dopaminergic neuronal death (Zhu et al. 2010; Zhu et al. 2014; Zou et al. 2013). Firstly, puerarin regulates proteasome activity to facilitate the degradation of ubiquitinated proteins and α-synuclein (Cheng et al. 2009). Secondly, puerarin possesses anti-oxidative activity to antagonize the radical oxidative stress and reverses the impairment of mitochondrial membrane potential to prevent apoptosis (Cheng et al. 2011). Finally, we found that puerarin promoted Akt phosphorylation, and this process prohibited p53 protein nuclear translocation (Zhu et al. 2012). In this study, we showed that puerarin functions via facilitating GPR30 expression. As reported in previous studies, activation of GPR30 could stimulate Akt phosphorylation to exert its biological action (Ge et al. 2013; Yang et al. 2016). We previously also reported that puerarin activated the Akt signaling pathway to prohibit MPP^{+}-induced cell death (Zhu et al. 2012). In this study, it is possible that puerarin activated Akt by facilitating GPR30, based on previous publication (Bourque et al. 2014). In addition, GPR30 also enhances melanogenesis via cAMP-protein kinase pathway (Sun et al. 2016).

Because puerarin has an estrogen-like structure, it could function by directly binding to GPR30 (Zhang et al. 2005; Zheng et al. 2002). Although we could not exclude whether puerarin acted as a direct agonist to GPR30 to exhibit its effect, its application increased GPR30 expression in both normal and MPP^{+}-treated SH-SY5Y cells. Moreover, new synthesis of GPR30 contributed to puerarin-mediated protection, as both rapamycin and protein synthesis inhibitors blocked this action. Concurrently, the mTor pathway was activated upon puerarin treatment, and this signaling pathway regulated protein synthesis. Using a system to detect the newly synthesized proteins, we found that puerarin application increased protein synthesis, including that of GPR30. Moreover, new synthesis of GPR30 was blocked by an mTor inhibitor (Briz et al. 2015b). To the best of our knowledge, this is the first report implicating that phytoestrogens could activate protein synthesis similar to E2 (Tuscher et al. 2016; Xing et al. 2015). Most importantly, MPP^{+}-induced cell death was mitigated by rapamycin application. Taken together, puerarin probably facilitates the Akt/mTor pathway to stimulate GPR30, and this process prevents MPP^{+}-induced cell death (Bourque et al. 2014).

The question of how new synthesis of GPR30 was initiated is still debatable. It is possible that puerarin functions through binding to endogenous GPR30 to elicit the synthesis of GPR30 and membrane insertion. Thus, a feedback loop might enhance the activity of GPR30. It is also possible that puerarin activates the Akt/mTor pathway and subsequently stimulates GPR30 expression. We could thus conclude that the new synthesis of GPR30 performs critical roles in preventing MPP^{+}-induced cell death. To clearly demonstrate these mechanisms, the endogenous GPR30 should be silenced to investigate the potential roles of endogenous GPR30.

GDNF is an important neurotropic factor. Several studies have shown its roles performed in regulating dopaminergic cell survival (d'Anglemont de Tassigny et al. 2015; Gao et al. 2016). Although several practical problems still exist regarding the viral vector delivery of neurotrophic factors for PD therapy (Kelly et al. 2015), adenovirus-, adeno-associated virus-, and lentivirus-mediated GDNF expressions were reportedly useful in PD models (Chen et al. 2014; Kells et al. 2010; Lu-Nguyen et al. 2014). In our previous study, we also found that GDNF expression was depleted in an 6-OHDA-induced PD model and in an MPTP-insulted model, and puerarin pre-treatment was able to reverse the decrease of GDNF in a PD model (Zhu et al. 2010). In this study, we further detected GDNF expression to observe the potential function of GPR30 in GNDF release. The up-regulation of GDNF is crucial for GPR30-mediated protection in PD (Bessa et al. 2015).

Conclusion

We showed a new puerarin-mediated protective mechanism in an MPP^+-induced PD model. Puerarin stimulated GPR30 synthesis to exhibit its neuroprotection through activating the mTor pathway. Moreover, GDNF was activated following GPR30 activation. These data implicated that GPR30 served as a potential target for PD treatment.

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Complaince with ethical standard

Conflict of Interest The authors declare that they have no conflict of interest.

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