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ORIGINAL ARTICLE

Quinolinic acid induces cell apoptosis in PC12 cells through HIF-1-dependent RTP801 activation

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Abstract Neurological disease comprises a series of disorders featuring brain dysfunction and neuronal cell death. Among the factors contributing to neuronal death, excitotoxicity induced by excitatory amino acids, such as glutamate, plays a critical role. However, the mechanisms about how the excitatory amino acids induce neuronal death remain elucidated. In this study, we investigated the role of HIF-1α (hypoxia inducible factor-1α) and RTP801 in cell apoptosis induced by quinolinic acid (QUIN), a glutamatergic agonist, in PC12 cells. We found that QUIN at 5 μM increased the expression of HIF-1α significantly with a peak at 24 h. After the treatment with QUIN (5–20 μM) for 24 h, the cells exhibited decreased viability and cell apoptosis with a concomitant increased expression of apoptosis related proteins. QUIN treatment also induced the generation of intracellular reactive oxygen species and RTP801 up-regulation in a HIF-1α-dependent manner that were inhibited by 2-methoxyestradiol, a HIF-1α inhibitor. Importantly, HIF-1 or RTP801 invalidation by siRNA rescued the cell apoptosis induced by QUIN or cobalt chloride, a chemical inducer of HIF-1. Taken together, these findings support the concept that neurotoxicity induced by QUIN is associated with HIF-1-dependent RTP801 activation and provide insight into the potential of RTP801 inhibitor in treatment of neurological disorders.

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Introduction

Excitotoxicity, the sustained activation of receptors for excitatory amino acids, plays an important role in the pathogenesis of various chronic neurodegenerative disorders, such as Parkinson's disease (PD) and Huntington's disease (HD). The excessive stimulation of glutamate receptors, mostly the N-methyl-D-aspartate (NMDA) subtypes, increases intracellular Ca^{2+} levels and triggers events for the initiation of deadly cascades (Mattson 2007; Szydlowska and Tymianski 2010). In addition, the biological changes have been featured in animal models of neuronal excitotoxicity, such as generation of reactive oxygen species (ROS), lipid peroxidation, inhibition of cellular respiration and elevated inflammation (Barger et al. 2007; Maalouf et al. 2007).

Quinolinic acid (QUIN), a selective NMDA receptor agonist, is a metabolite of tryptophan along the kynurenine pathway (Lugo-Huitron et al. 2013). In neurons, sustained NMDA receptor activation by QUIN induces Ca^{2+} influx and subsequent activation of proteases, phospholipase and endonucleases, resulting in neuronal death (Ryu et al. 2005; Essa et al. 2013). QUIN also induced the autophagy of neuronal cells, which can be blocked by NMDA antagonist MK801 (Braidy et al. 2014). In glial cells, QUIN enhances the expression and secretion of potent chemokines and proinflammatory cytokines, such as interleukin-1β, monocyte chemoatractant protein-1 and interferon-γ (Guillemin et al. 2005), leading to marked inflammatory responses in brain. In vivo studies have shown that intrastriatal administration of QUIN in rodents produces similar biochemical changes to that in patients with HD (Pierozan et al. 2014). In patients with neurodegenerative disorders, the content

of QUIN in brain is also elevated (Stoy et al. 2005). These results suggest the role of QUIN in the pathology of neurodegenerative disorders, while the mechanisms by which QUIN induces neuronal degeneration and loss still remain elucidated.

Increasing evidences show that excitotoxins induce neuronal apoptosis, leading to neurodegeneration (Mehta et al. 2013). Among the factors involved in neuronal apoptosis, hypoxia inducible factor-1 (HIF-1), a transcription factor that mediates fundamental cellular responses to hypoxia, regulates the adaptive response of neuronal cells to different stresses (Semenza 2000, 2010). HIF-1 is composed of two subunits, an oxygen-regulated HIF-1α and a constitutively expressed HIF-1β subunit. During hypoxia, HIF-1α is stabilized, dimerizes with HIF-1β and translocates to the nucleus to bind to hypoxia responsive elements (Semenza 2010). Thereafter, multiple target genes of HIF-1 will be activated, including glycolytic enzymes, growth factor and proapoptic proteins (Zaman et al. 1999; Meijer et al. 2012; Ferrer et al. 2014). Under ischemic conditions, HIF-1α is significantly up-regulated in neuronal cells and exerts regulatory effects on the tissue injury (Helton et al. 2005; Baranova et al. 2007). Since HIF-1α is involved in both protective signals and detrimental changes, the role of HIF-1α in neuronal survival remains controversial.

Among the target genes of HIF-1 involved in regulation of cell survival, RTP801 (REDD1/DITT4) functions as a suppressor of mammalian target of rapamycin (mTOR), resulting in the inhibition of mTOR activity following modest hypoxia or energy deprivation (Brugarolas et al. 2004). In response to hypoxia, RTP801 is highly induced in Drosophila and mammalian cells (Reiling and Hafen 2004; DeYoung et al. 2008). Overexpression of RTP801 potently inhibits the activity of mTOR, whereas genetic deletion of RTP801 impairs the down-regulation of mTOR activity in hypoxia (DeYoung et al. 2008). Moreover, RTP801 has been shown to function in regulation of PI3K/Akt signaling and formation of intracellular reactive oxygen species (ROS) (Regazzetti et al. 2010), implying multiple roles of RTP801 in cellular physiological process.

In this study, we investigated the effects of QUIN on cultured rat adrenal pheochromocytoma PC12 cells. We demonstrated that QUIN induces HIF-1α accumulation and activation, leading to the activation of RTP801 and cell apoptosis. The results indicate that inhibition of HIF-1α or RTP801 may be a potential neuroprotective strategy to counteract the toxicity of QUIN and an efficient therapy for neurodegenerative disease.

Results

QUIN induced PC12 cell injury, ROS generation and HIF-1α up-regulation

We first determined the cell viability of PC12 cells by MTT assay after the treatment with different concentrations of QUIN for 24 h. As shown in Fig. 1a, QUIN (2.5–20 μM) reduced cell viability in a concentration-dependent manner. In addition, administration of QUIN at 2.5 ~ 10 μM for 2 h significantly induced intracellular ROS generation (Fig. 1b).

It has been reported that after the stimulation by ischemia or glutamate, the stabilization of HIF-1α is significantly induced in neurons, leading to the cell death (Helton et al. 2005). Thus, to characterize the expression of HIF-1α in response to QUIN, a time course of induction curve was performed. PC12 cells were treated with QUIN for increased periods of time, and HIF-1α expression was detected by immunoblotting. As shown in Fig. 1c, HIF-1α was weakly expressed in PC12 cells under normal conditions. An increase in HIF-1α protein level was detected after a 0.5-h incubation with QUIN at 5 μM, the level peaked at 24 h and declined to basal level at 72 h.

Pharmacological Inhibition of HIF-1α protected PC12 cells against QUIN-induced cell injury

Employing 2-methoxyestradiol (2-Me), a HIF-1α inhibitor, we assessed the cell viability and HIF-1 expression. As shown in Fig. 2, 2-Me at 10 μM significantly protected PC12 cells against the injury induced by QUIN treatment at 5 μM for 24 h, while 2-Me at 10 μM alone did not affect the cell viability (data not shown).

During the neuronal death induced by neurotoxin, apoptotic cell death accounted for a large part of neuronal death. Thus, we determined the cell apoptosis induced by QUIN and the effect of HIF-1α inhibitor on cell apoptosis. Using Hoechst 33258 staining, we found that PC12 cells underwent apoptosis after the treatment with QUIN for 24 h, showing condensed nuclei and enhanced blue fluorescence (Fig. 3a). The apoptosis was also blocked by 2-Me at 10 μM.

The apoptosis regulator proteins, such as B-cell lymphoma 2 (Bcl-2) and bcl-2-like protein 4 (Bax), are involved in the modulation of cell apoptosis. Bcl-2 possesses an anti-apoptotic activity, while Bax is a pro-apoptotic protein and the increased expression ratio of Bax and Bcl-2 indicates the execution of apoptosis (Rahmani et al. 2013). After the QUIN treatment at 5 ~ 10 μM for 24 h, the expression ratio of Bax/Bcl-2 was increased, which was blocked by the administration of 2-Me at 10 μM (Fig. 3b).

Treatment with 2-Me decreased blocked HIF-1α up-regulation and nucleic accumulation

To confirm that the protective effect of 2-Me is due to HIF-1α inhibition, PC12 cells were treated with QUIN at 5 μM for 24 h with or without 2-Me. As shown in Fig. 4a, 10 μM of 2-Me blocked the HIF-1α up-regulation after QUIN treatment. Upon activation, HIF-1α is transferred into nuclei and functions as a potent transcriptional factor, initiating the expression

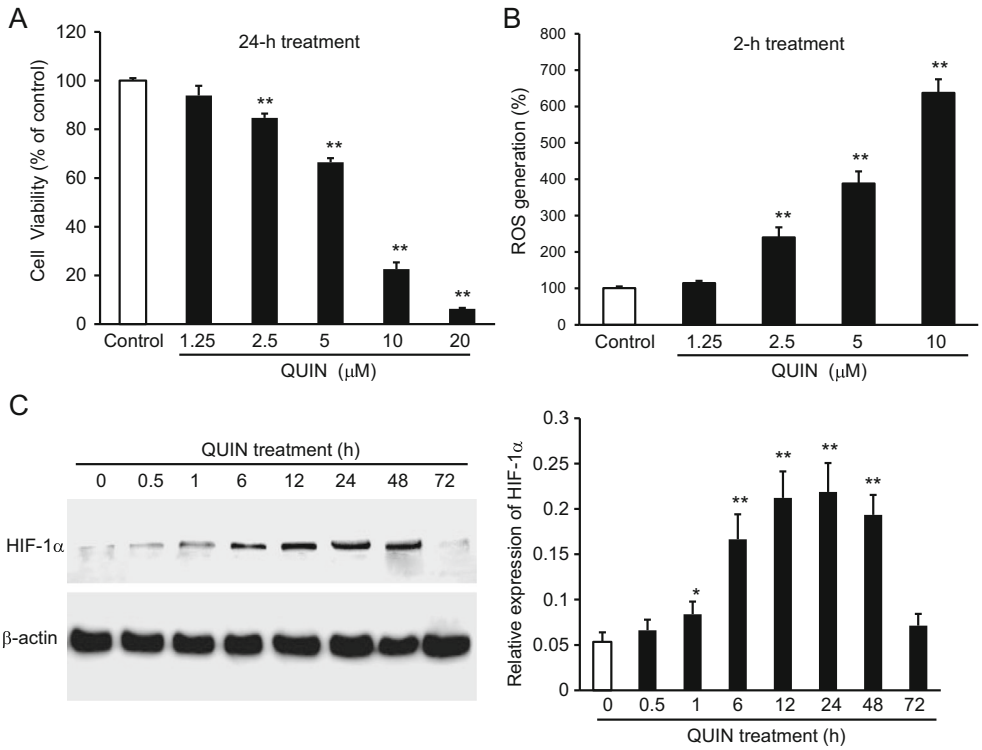

Fig. 1 QUIN decreased cell viability, increased ROS generation and HIF-1α expression in PC12 cells. **a** The treatment with QUIN for 24 h reduced the cell viability determined by MTT reduction assay. **b** ROS generation was significantly induced in PC12 cells by QUIN. **c** Western blotting analysis showed the increased expression of HIF-1α in PC12 cells treated with QUIN. Data are expressed as mean ± SD; $n = 8$ wells for cell viability and ROS generation, $n = 4$ for HIF-1α expression; $^{*}P < 0.05$, $^{**}P < 0.01$ compared with control

of its target genes (Semenza 2000). By immunostaining with HIF-1α antibody, we found that HIF-1α was weakly expressed in cytosol under normal conditions. While after the treatment with QUIN, HIF-1α protein expression was markedly induced

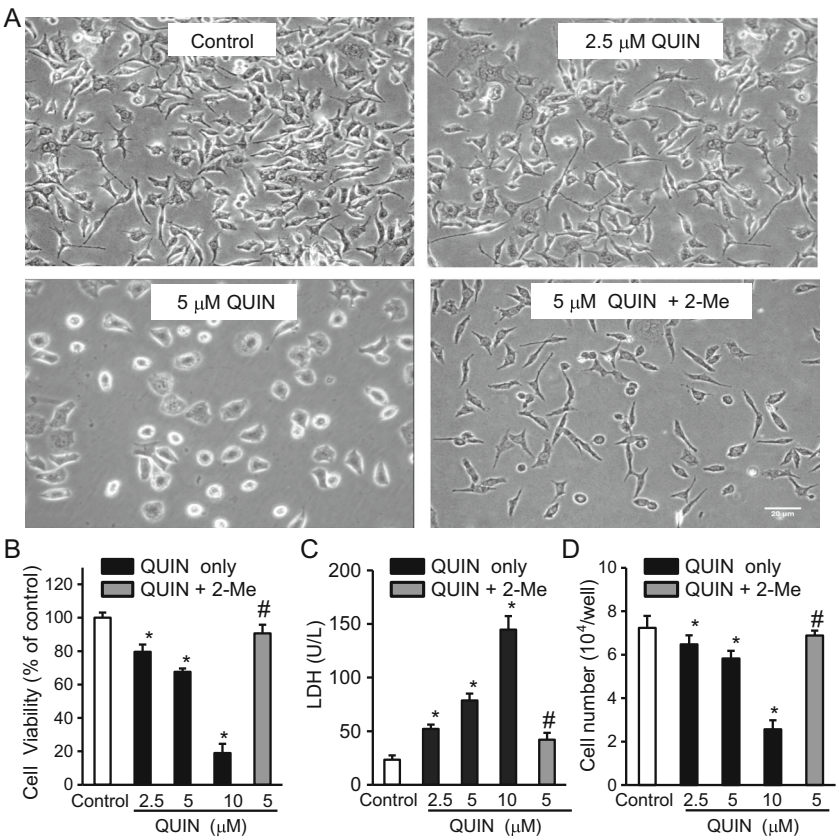

Fig. 2 A HIF-1α inhibitor 2-Me protected the PC12 cells from cell injury induced by QUIN. **a** Representative micrographs showed the morphological changes in PC12 cells at 24 h after QUIN treatment. Scale bar = 20 μm. **b** The pretreatment with 2-Me attenuated cell injury induced by QUIN treatment for 24 h. **c** and **d** The pretreatment with 2-Me protected against cell death detected by LDH activity measurement and cell counting. Data are expressed as mean ± SD; $n = 8$; $^{*}P < 0.01$ compared with control, $\#P < 0.01$ compared with 5 μM QUIN

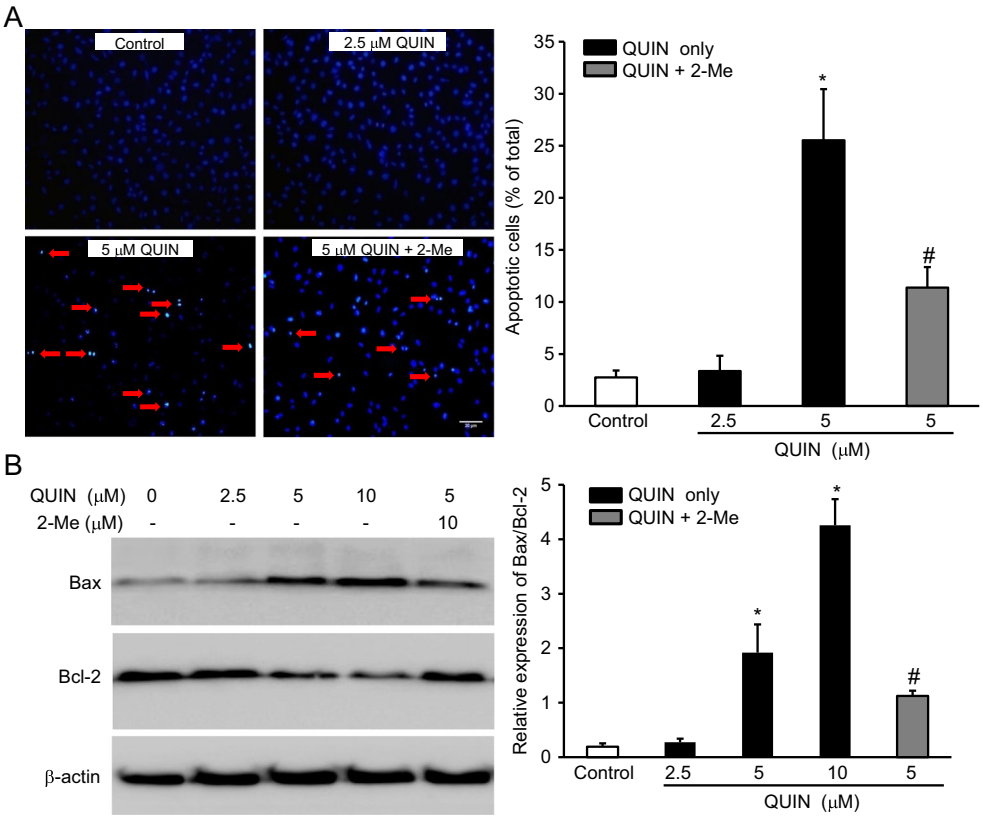

Fig. 3 2-Me attenuated cell apoptosis induced by QUIN. **a** Representative micrographs showed the apoptotic cells at 24 h after QUIN administration with or without 2-Me. Arrows indicate apoptotic cells exhibiting condensed nuclei with strong bright Hoechst 33258 staining. Scale bar = 20 μm. **b** 2-Me decreased Bax expression and increased Bcl-2 expression, resulting in the reduced ratio of Bax and Bcl-2. Data are expressed as mean ± SD; $n = 4$; $^{*}P < 0.01$ compared with control, $\#P < 0.01$ compared with 5 μM QUIN

and translocated into the nuclei (Fig. 4b). Accordingly, 2-Me blocked the nuclear accumulation of HIF-1α.

Knockdown of HIF-1α blocked the induction of RTP801

Given the biological importance of RTP801 in cell apoptosis and the fact that RTP801 is a downstream target of HIF-1α, we speculated that HIF-1α/RTP801 signaling might play a role in PC12 cell injury. In order to efficiently inhibit HIF-1α and RTP801 expression, PC12 cells were transfected with siRNA directed against HIF-1α and RTP801. As shown in Fig. 5a, HIF-1α siRNA significantly inhibited HIF-1α and RTP801 induction by QUIN. However, knockdown of RTP801 only blocked RTP801 up-regulation but did not affect HIF-1α expression. Likewise, quantitative PCR analysis showed that HIF-1α siRNA abolished the up-regulation of both HIF-1α and RTP801 mRNA, and RTP801 siRNA only inhibited the induction of RTP801 (Fig. 5b).

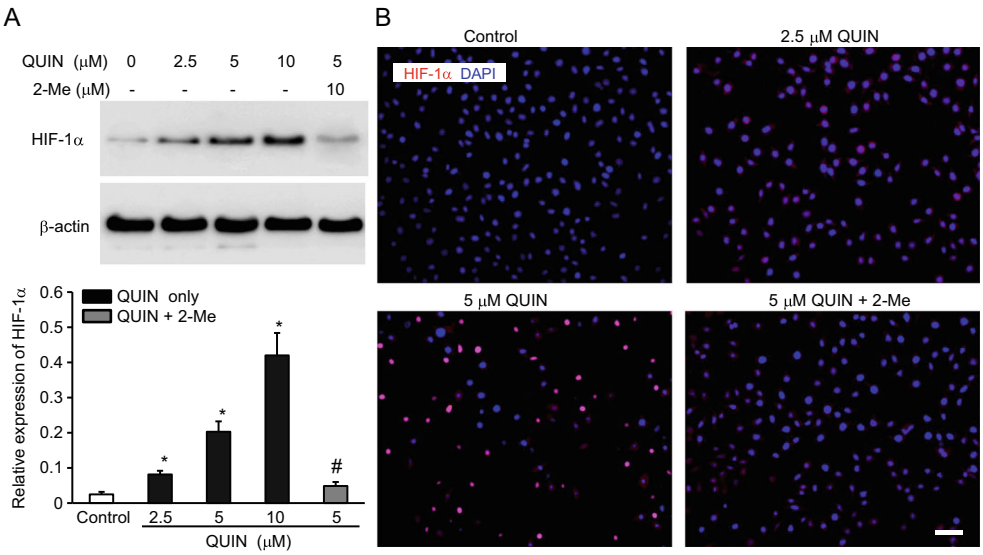

Fig. 4 2-Me inhibited HIF-1α activation and up-regulation induced by QUIN. **a** Treatment with 2-Me blocked HIF-1α up-regulation. Data are expressed as mean ± SD; $n = 4$; $^{*}P < 0.01$ compared with control, $\#P < 0.01$ compared with 5 μM QUIN only. **b** Representative micrographs showed that 2-Me inhibited the nucleic accumulation of HIF-1α induced by QUIN. Scale Bar = 20 μm

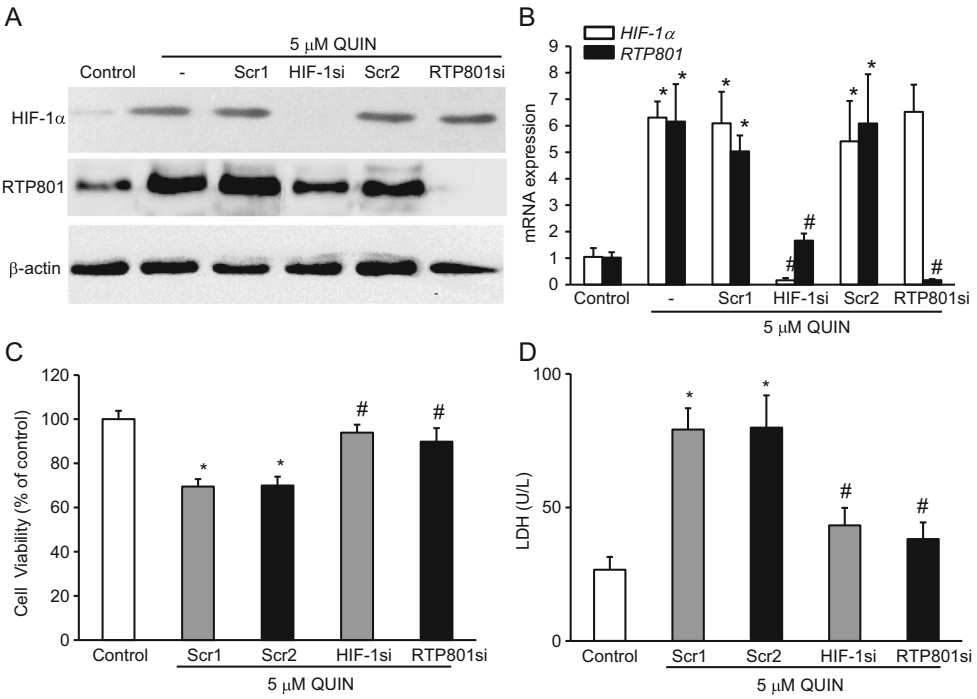

Fig. 5 Silencing of HIF-1α or RTP801 rescued PC12 cell death induced by QUIN. **a** Western blotting analysis demonstrated that HIF-1α or RTP801 siRNA blocked the up-regulation of HIF-1α and RTP801 at 24 h post-QUIN challenge. Results are representative of 3 separate experiments. **b** Quantitative PCR analysis showed that HIF-1α or RTP801 siRNA inhibited HIF-1α or RTP801 up-regulation. Scr1: HIF-1α scrambled RNA, scr 2: RTP801 scrambled RNA. **c and d** Silencing of HIF-1α or RTP801 protected the PC12 cells against cell injury. Data are expressed as mean ± SD; $n = 4$; $^{*}P < 0.01$ compared with control, $\#P < 0.01$ compared with QUIN only

Knockdown of HIF-1-dependent RTP801 rescued QUIN-induced cell injury

Previous studies have showed that RTP801 is activated following HIF-1α activation, resulting in the inhibition of mTOR signaling (Horak et al. 2010). Subsequently, the activation of RTP801 leads to a series of biochemical processes, such as cell apoptosis, cell growth arrest, and autophagy (Schwarzer et al. 2005; Ben Sahra et al. 2011). Thus, we determined the effect of HIF-1α or RTP801 siRNA on cell death induced by QUIN. As shown in Fig. 5c and d, the apoptotic cell death induced by QUIN was rescued by either HIF-1α or RTP801 siRNA (Fig. 6a). In addition, employing HIF-1α and RTP801 siRNA, the expression ratio of Bax/Bcl-2 was reduced (Fig. 6b). To confirm the involvement of HIF-1α/RTP801 signaling, PC12 cells were treated with cobalt chloride, a chemical inducer of HIF-1. As shown in Fig. 7a, b and c, the apoptotic cell death induced by cobalt chloride was attenuated by HIF-1α or RTP801 siRNA. Exposure of the cells to cobalt chloride for 4 h induced significant elevation in HIF-1α and RTP801 protein levels, which were blocked by HIF-1α or RTP801 siRNA respectively (Fig. 7d).

Discussion

In this study, we found that QUIN at 5 ~ 20 μM significantly induced cell apoptosis and HIF-1α up-regulation in PC12 cells. Pharmacological inhibition of HIF-1α by 2-Me blocked the nuclear accumulation of HIF-1α and the cell injury. In addition, RNA invalidation of HIF-1α by siRNA blocked RTP801 expression; both HIF-1α and RTP801 siRNA protected the cells against apoptosis, suggesting that the HIF-1α-dependent RTP801 activation is involved in the cell injury. These results are consistent with the previous results that QUIN induces neuronal apoptosis in vivo (Nakai et al. 1999; Colin-Gonzalez et al. 2013) and confirmed the role of RTP801 in neuronal apoptosis.

In addition to persistent stimulation of NMDA receptor, elevated ROS generation also contributes to the excitotoxicity by QUIN. In the presence of oxygen, QUIN produces the accumulation of metal ions, such as Cu^{2+} and Fe^{2+}, and therefore increases the formation of superoxide anion, hydrogen peroxide and hydroxyl radical (Colin-Gonzalez et al. 2013). Thereafter, increased ROS formation leads to the DNA injury, lipid peroxidation and damage of biomembrane structure and neuronal cell apoptosis (Duong et al. 2008). Recently, it has been reported that ROS can also induce the activation of HIF-1α (Yuan et al. 2011) and modulate neuronal apoptosis (Agrawal et al. 2011). In neuronal cells, ischemic-like insult induces ROS generation, HIF-1α activation and cell apoptosis, which can be reversed by antioxidants (Rayner et al. 2006; Duong et al. 2008). Consistent with the previous studies, our study showed that PC12 cells exhibited elevated ROS level and HIF-1α expression after QUIN treatment, which was accompanied by cell apoptosis, implying the role of ROS/HIF-1α pathway in neuronal death.

A series of studies have been performed to elucidate the molecular mechanisms by which HIF-1α regulates neuronal survival and suggest that HIF-1α may possess opposite effects

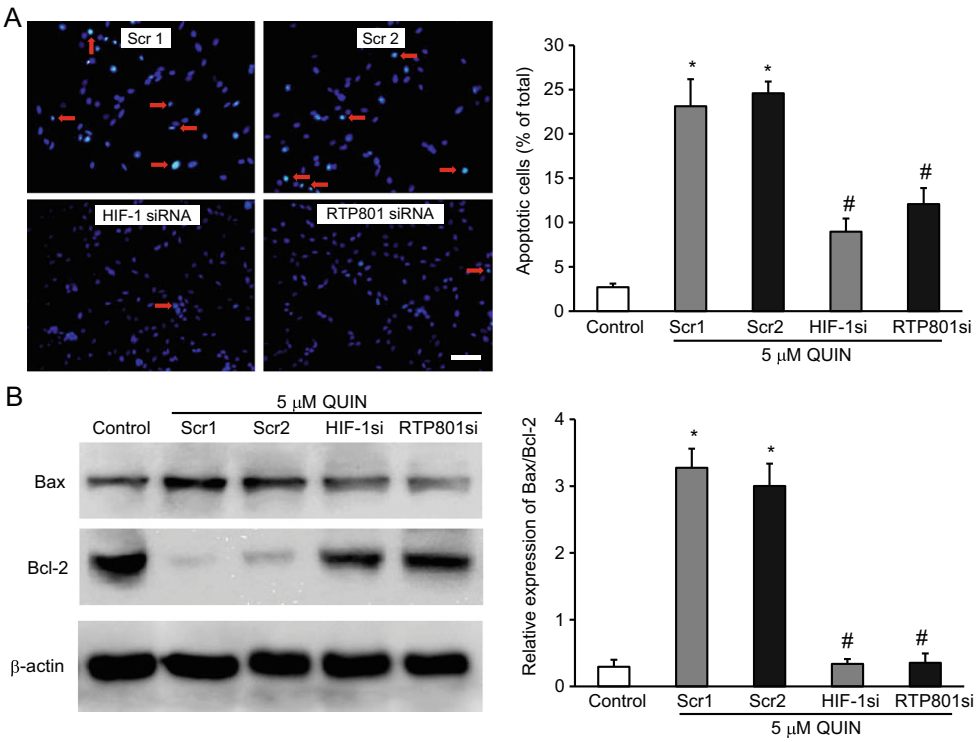

Fig. 6 Silencing of HIF-1α or RTP801 blocked cell apoptosis in the PC12 cells after QUIN treatment. **a** Hoechst 33258 staining assay demonstrated that HIF-1α or RTP801 siRNA inhibited the cell apoptosis. **b** Knockdown of HIF-1α or RTP801 decreased the expression ratio of Bax and Bcl-2. Data are expressed as mean ± SD; $n = 4$; $^*P < 0.01$ compared with control, $\#P < 0.01$ compared with the respective scrambled RNA

in different conditions. For example, inhibition of HIF-1α protects cortical neurons against ischemic insult (Lin et al. 2013; Cheng et al. 2014) and activation of HIF-1α contributes to neuronal apoptosis (Jiang et al. 2012), indicating the pro-apoptotic character of HIF-1α. In severe hypoxic conditions, HIF-1 activation potentiates p53 signaling and leads to neuronal apoptosis (Fan et al. 2009). While other studies show the protective effects of HIF-1 activation on neuronal death.

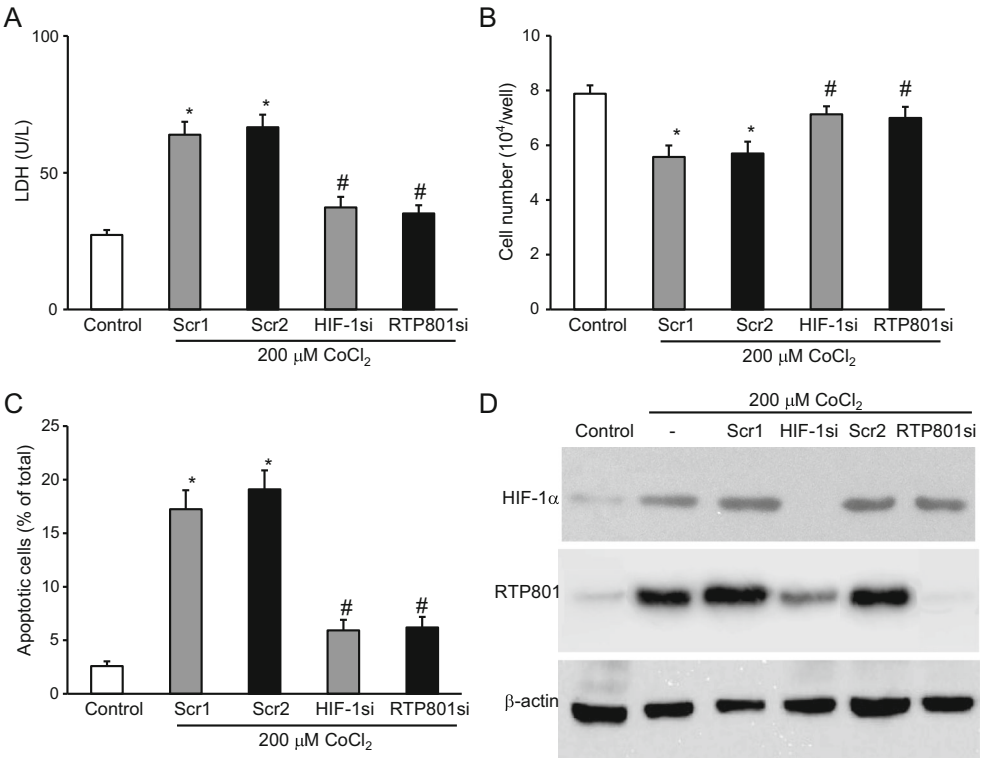

Fig. 7 Knockdown of HIF-1α or RTP801 attenuated cell apoptosis induced by cobalt chloride. **a and b** The treatment with HIF-1α or RTP801 siRNA attenuated cell death induced by cobalt chloride. **c** Cell apoptosis was ameliorated by knockdown of HIF-1α or RTP801 expression. **d** After 4-h incubation of cobalt chloride, the expression of HIF-1α and RTP801 was markedly increased. HIF-1α or RTP801 siRNA blocked the up-regulation of HIF-1α and RTP801 respectively. Data are expressed as mean ± SD; $n = 4$; $^*P < 0.01$ compared with control, $\#P < 0.01$ compared with the respective scrambled RNA

Inhibition of HIF-1 abolishes the beneficial effects of the neuroprotective agent (Lopez-Hernandez et al. 2015) and enhances the toxicity of neurotoxins (Jeong and Park 2012), whereas induction of HIF-1 protects against neuronal death (Seo et al. 2010; Du et al. 2011). HIF-1 may also up-regulate the expression of growth factors and exert anti-apoptotic effects (Piret et al. 2002). Our results showed that HIF-1α expression was increased after the treatment with QUIN from 6 h with a peak at 24 h and the nucleic accumulation of HIF-1α was induced. Both the upregulation and nucleic accumulation of HIF-1α were blocked by 2-Me. These results support the detrimental role of HIF-1α in neuronal survival and suggest that pharmacological inhibition of HIF-1α may be a useful therapy for neuronal excitotoxicity.

Several signaling pathways are involved in QUIN-induced cell death, including nuclear factor-like 2 (Colin-Gonzalez et al. 2014), peroxisome proliferator activated receptor-γ (Mishra et al. 2014), and histone deacetylase (Mishra et al. 2014). In this study, we found that RTP801 was involved in mediating the QUIN-induced toxicity, demonstrated by protection against cell death by the knockdown of RTP801 expression. RTP801 has been reported to be related to ROS generation and DNA damage. In an animal model of cerebral ischemia, RTP801 expression is increased in the infarct region (Wu et al. 2011). In patients with PD and HD, the increased levels of RTP801 are also detected in neurons (Malagelada et al. 2006; Martin-Flores et al. 2015). Recently, RTP801 has been identified as a negative regulator of Schwann cell myelination and knockdown of RTP801 produced more myelinated segments (Noseda et al. 2013). Additionally, the up-regulation of RTP801 is shown in animal models of non-neuronal injury, such as cardiac ischemic injury and acute cigarette smoke–induced lung injury (Yoshida et al. 2010). Taken together, these results identify RTP801 as a positive regulator of neuronal death.

In addition, we found that silencing of HIF-1α expression by siRNA reduced RTP801 expression and protected cells against cell injury induced by QUIN, indicating the involvement of HIF-1α/RTP801 signaling in neuronal death. To confirm the role of HIF-1α/RTP801 signaling in cell death, the cells were treated with cobalt chloride. Similar to QUIN treatment, cobalt chloride induced the up-regulation of HIF-1 and RTP801 and cell apoptosis that was rescued by knockdown of HIF-1α or RTP801. In agreement with our results, knockdown of RTP801 exhibits neuroprotection in the animal models of HD and ischemic injury (Shoshani et al. 2002; Martin-Flores et al. 2015). Inhibition of RTP801 by rapamycin or siRNA also protects against neuronal death in experimental models of PD (Malagelada et al. 2010). These results confirm that inhibition of RTP801 may be useful in the treatment of ischemic disease or neurodegenerative disease. Importantly, we found that HIF-1α siRNA blocked the expressions of HIF-1α and RTP801, while RTP801 siRNA cannot block HIF-1α up-regulation, showing that HIF-1α is the upstream signal of RTP801.

In summary, our studies demonstrate that QUIN induced intracellular ROS generation and HIF-1α activation in rat PC12 cells, leading to HIF-1α-dependent RTP801 activation and cell apoptosis in rat PC12 cells. Blockade of HIF-1α or RTP801 expression prevents QUIN-induced death. Hence, RTP801 is a downstream effector of HIF-1α and pharmacological inhibition of RTP801 might be a potential approach for the treatment of neurodegenerative disease.

Experimental procedures

Cell culture

Rat pheochromocytoma cells (PC12 cells) were purchased from Institute of Cell Biology, Chinese Academy of Science (Shanghai, China). The cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Gibco Life Technologies, USA) supplemented with 10 % horse serum, 5 % fetal bovine serum, penicillin (100 000U/L) and streptomycin (100 mg/L; Sigma-Aldrich Chemical Co., MO, USA) and maintained in a humidified atmosphere at 37 °C. The cells were differentiated with 50 ng/ml nerve growth factor (NGF, #0005017, Harlan Laboratories Inc, USA) in DMEM with 1 % fetal bovine serum for 9 days. Thereafter, the cells were washed with DMEM at 24 h before experiments and cultured in DMEM with 1 % fetal bovine serum. In the experiments involving treatment with drugs, cells were pre-treated for 30 min with drug or vehicle. The stock solutions of cobalt chloride (200 mM; Sigma-Aldrich Chemical Co., USA) and 2-methoxyestradiol (20 mM; Sigma-Aldrich Chemical Co., USA) were prepared before each treatment.

Cell death determination

Cell injury was examined by the determination of lactate dehydrogenase (LDH) level in culture medium. Fifty microliters of culture supernatants were collected from each well and LDH activity was measured by a LDH assay kit (Roche, USA) according to the manufacturer's instruction.

Cell viability assay

Cells were plated at 5×10^4/ml in 96-well plates. After 24 h, cells were treated with QUIN and the agents. After the treatment, 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, USA) was added to each well to reach a final concentration of 0.5 mg/ml. After incubation at 37 °C for 4 h, the medium was removed and 100 μl dimethyl sulfoxide was added to each well. The absorbance at 490 nm was measured with a microplate reader (Elx800, Bio-Tek

instrument, USA). Results were expressed as the percentage of control. In another series, at the end of the treatment, the cells were trypsinized and resuspended in the medium mixed with 0.4 % trypan blue (1:1, Sigma-Aldrich, USA). The trypan blue negative cells were counted using a haemocytometer.

Intracellular reactive oxygen species determination

The measurement of intracellular reactive oxygen species (ROS) was based on the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA, Sigma-Aldrich, USA) to an intracellular fluorescent product. Briefly, after the treatment with QUIN for 2 h, PC12 cells grown on 96-well plates were washed with Hank's solution and incubated with 50 μM H_2DCFDA in Hank's solution for 40 min. Thereafter, the cells were washed and the fluorescence was measured at excitation of 485 nm and emission of 530 nm on a platereader (Varioskan Flash, ThermoFisher Scientific Inc, USA).

Cell apoptosis assay

Cells grown on coverslips were washed with PBS and then stained with Hoechst 33258 at 10 mg/L for 10 min at 37 °C. thereafter, the cells were observed under a fluorescent microscope (Olympus BX41, Japan). The apoptotic cells were determined as condensed or fragmented nuclei with strong bright fluorescence. At least 10000 cells were counted in more than 4 fields in each coverslip. The apoptotic cells were expressed as percentage of total cells.

Immunocytochemistry for the detection of HIF-1α

Cells seeded on coverslips were fixed with ice-cold methanol for 5 min and incubated in 5 % normal goat serum for 1 h at room temperature. Then the cells were incubated with mouse monoclonal anti-HIF-1α antibody (1:100, Novus, USA) at 4 °C overnight. After washing with PBS, the coverslips were incubated with Alexa Fluor 488-conjugated secondary antibody (1:600, Jackson ImmunoResearch Laboratories, USA). The nuclei were counterstained with DAPI (4′,6-diamidino-2-phenylindole). Finally, the labeled cells were observed with a fluorescent microscopt (BX-41, Olympus, Japan).

Quantitative PCR

Total RNA was isolated using an RNeasy Mini kit including DNase I digestion (Qiagen, USA). The reverse transcription reaction was carried out with a High Capacity cDNA Archived Kit (Applied Biosystems, USA) according to the manufacturer's protocol. Subsequently, the real-time PCR analysis was performed with a sequence detection system (ABI Prism 7000; Applied Biosystems, USA). Amplification of specific PCR products was detected using the SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer's protocol. The following primers were used for analysis: rat HIF-1α, 5′-CCA CAGGACAGTACAGGAG-3′ and 5′-TCAAGTCGT GCTGAATAATC; rat RTP801, 5′-GCTCTGGAC CCCAGTCTAGT-3′ and 5′-GGGACAGTCCTT CAGTCCTT-3′; rat cyclophilin 5′-CCCACCGTGTTCT TCGACAT-3′ and 5′-TGCAAACAGCTCGAAGCAGA-3′. The gene expression was normalized to cyclophilin.

Western blotting analysis

The cells were washed twice with ice-cold PBS and then lysed in cell lysis buffer (Cell Signaling Technology, USA) containing 1 μM phenylmethysulfonyl fluoride (Sigma-Aldrich, USA) at 4 °C. Then the homogenates were centrifuged at 10,000×*g* for 30 min at 4 °C. The protein samples were separated by a 10 % SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane (Millipore, USA). The blot was blocked with 5 % non-fat milk and incubated with an anti-HIF-1α antibody (1:500, Novus Biologicals, USA), an anti-RTP801 antibody (1:1000, Thermo Fisher Scientific Inc., USA), an anti-Bax antibody (1:2000, Cell Signaling Technology, USA), an anti-B-cell lymphoma 2 antibody (Bcl-2, 1:2000, Cell Signaling Technology, USA) and an anti-beta-actin antibody (β-actin, 1:2000, Cell Signaling Technology, USA) at 4 °C. Overnight primary antibody incubation was followed by incubation with a horseradish-conjugated secondary antibody (1:5000, Jackson ImmunoResearch Laboratories, USA) and enhanced chemiluminescence reagents (Pierce Biotechnolog, USA). Blots were exposed on an X-ray film. The results of protein expression are normalized to β-actin.

siRNA treatment of cells

Cells were grown to 60 % confluence in 24 well plate before transfection and 20 pmol of duplex siRNA (Santa Cruz Biotechnology Inc, USA) were diluted in 200 μl of Opti-Mem I (Invitrogen life Technologies, USA). In parallel, 2 μl of Oligofectamine (Invitrogen life Technologies, USA) were added to 200 μl of Opti-Mem I and incubated at room temperature for 10 min. Then the indicated duplex siRNA oligonucleotide solution was added to the Oligofectamine/Opti-Mem I mixture, incubated at room temperature for 20 min. After rinse with Opti-Mem I to remove any residual serum, the cells were incubated with the siRNA complexes in serum-free conditions for 4 h at 37 °C in a 5 % CO_2 incubator. Serum was then added back to the medium, and cells were incubated for an additional 48 h before beginning an experiment.

Data analysis

Differences between groups were examined for statistical significance using one-way analysis of variance (ANOVA) followed by Dunnett's Multiple Comparison. $P<0.05$ denoted the presence of a statistically significant difference.

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Compliance with ethical standards

Conflict of interest The authors have declared that no conflict of interest exists.

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