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ORIGINAL PAPER

Pretreatment-Etidronate Alleviates $CoCl_2$ Induced-SH-SY5Y Cell Apoptosis via Decreased HIF-1α and TRPC5 Channel Proteins

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Abstract

Chronic hypoxic damage is one of the most common pathogenic factors that can cause neurodegenerative disorder in most cases. Etidronate (Eti) is one of the best-known earlier-generations of bisphosphonate derivatives for the treatment of bone-loss related diseases. Building on the preceding study of our laboratory, we found that Eti showed neuroprotective effects against 2-vessel occlusion induced vascular dementia (VD) in rats. Therefore, in this study, we attempted to elucidate the mechanism of action, which Eti protected cells from chronic hypoxic damage caused by $CoCl_2$ in SH-SY5Y cells in vitro. Our data showed that the pretreatment with 100 μM Eti partially improved hypoxic damage in cell viability and reduced the hypoxia-inducible factor-1α (HIF-1α) expression, which indicated chronic hypoxic level. Furthermore, the protein expression of TRPC5 channel and its mediated intracellular calcium ion concentration ($[Ca^{2+}]_i$) were decreased. In addition, the apoptosis-related proteins caspase-9, and caspase-3 as well as calcium/calmodulin-dependent protein kinase II (CaMK-II) were down-regulated after treatment with Eti. In conclusion, Eti shows neuroprotective effects on SH-SY5Y cells injured by $CoCl_2$ through resisting apoptosis caused by calcium influx, which may be related to the inhibition of HIF-1α protein and the decreased TRPC5 channel protein.

Keywords Etidronate · TRPC channel · SH-SY5Y cells · Neuroprotection · Chronic hypoxic damage

Abbreviations

Eti	Etidronate
VD	Vascular dementia
HIF-1α	Hypoxia-inducible factor-1α
CaMK-II	Calcium/calmodulin-dependent protein kinase II
BPs	Bisphosphonates
TRP	Transient receptor potential
SH-SY5Y cells	Human neuroblastoma cells
$CoCl_2$	Cobalt chloride;

Introduction

Hypoxia is one of the most common pathogenic factors for vascular dementia, which is threatening the human health [1]. And neurons are strongly depended on aerobic metabolism to supply energy, and particularly sensitive to oxygen. Therefore, people worked in special environments such as highlands, high altitude and deep sea, are easy to be hurt by hypoxia. Hypoxia will cause apoptosis, structure change and functional decline once the nerve cells are suffering from low oxygen [2–4]. These changes are closely related to vascular dementia, epilepsy, Alzheimer's, Parkinson's and other diseases [5, 6]. It is very important to look for the ways to prevent hypoxic damage for the prevention and treatment of clinical disorders and the health of human beings.

Bisphosphonates (BPs) are a group of clinical drugs to treat metabolic bone diseases, which were discovered in 1960s and mainly used in the treatment of osteoporosis, deformation osteitis, hypercalcemia and bone pains caused by malignant tumors [7]. As a BPs derivative, Etidronate (Eti) can treat not only various bone diseases, but also neurodegenerative disorders. The chemical structure of Eti was showed in Fig. 1. In previous study, Eti can rescue PC12

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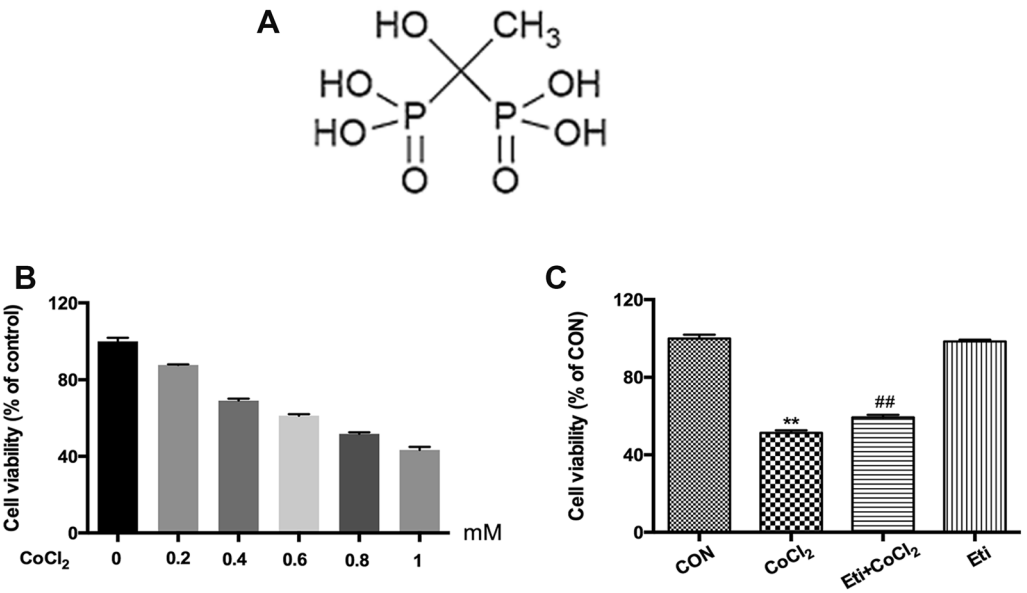

Fig. 1 Results of SH-SY5Y cell viability were analyzed by MTT. **a** Chemical structure of Eti. **b** SH-SY5Y cells were exposed to 0.2–1 mM $CoCl_2$ for 24 h. **c** SH-SY5Y cells were pretreated with 100 uM Eti for 12 h and then exposed to 0.8 mM $CoCl_2$. All data were expressed as mean ± SEM, n = 3. **p < 0.01 comparison between CON group and $CoCl_2$ group; ##p < 0.01 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

cells from excitability toxicity caused by glutamic acid through regulating the intracellular concentration of Ca^{2+} [8]. In addition, Eti can affect intracellular Ca^{2+} homeostasis to improve the synaptic transmission and inhibit oxidative stress and apoptosis to relieve the cognitive impairment in 2VO model rats [9]. These studies suggested that Eti regulated the intracellular Ca^{2+} homeostasis, which improved nerve injury as a kind of new potential neuroprotective drug.

The transient receptor potential (TRP) gene was identified in Drosophila, where mutants for the gene induced visual impairment since the cation Ca^{2+} influx pathway was blocked. Since then, investigators begin to pay close attention to TRP channels. The homologs gene was discovered in mammalian and referred to as TRPC1, TRPC2, and TRPC3 at first [10, 11]. Since then, the TRPC channel subfamilies (TRPC1–7) have gradually been found in mammalian cells including TRPC1, TRPC2, TRPC3/6/7, and TRPC4/5, which play a crucial role in intracellular Ca^{2+} homeostasis and the pathogenesis of several human diseases [12]. The TRPCs are broadly expressed in almost every tissue and cell type, especially in the nervous system [10, 11], which are activated by various mechanisms to effect the various cell functions.

Building upon the preliminary studies, we suspected that Eti could regulate the TRPC channel activity and improved nerve damage acting as a nerve protective drug.

Human neuroblastoma cells (SH-SY5Y cells) are very similar to neurons in structure and function, which take the place of neurons in our experiment [13–16].

Cobalt ions are one of the critical chemical inducers of hypoxia-like responses [17–19]. $CoCl_2$ often make chronic hypoxia model in vitro at home and abroad because of easy to use and stable of physical and chemical properties. In the previous study, it has suggested that $CoCl_2$ can induce hypoxic damage in PC12 cells, myocardial cells, endothelial cells and other cells in vitro [20, 21]. Similarly, $CoCl_2$ can also induce hypoxic/ischemic conditions, for example, the generation of reactive oxygen species and transcriptional changes in some genes, such as hypoxia-inducible factor-1α (HIF-1α), p53, p21, and PCNA [19, 22, 23]. Above all, $CoCl_2$-induced apoptosis may be a simple and convenient in vitro model for investigating the molecular mechanisms in hypoxia-induced cell death. Therefore, $CoCl_2$ was used to make chronic hypoxia damage model in our experiment.

Based on the above, we aim to clarify whether pre-Eti has neuroprotective effects on SH-SY5Y cells injured by $CoCl_2$ and the mechanism is achieved by regulating the TRPC channel.

Materials and Methods

Chemicals

Dulbecco modified Eagle's media (DMEM) high glucose was purchased from Hyclone. Fetal bovine serum (FBS) was purchased from Gibco, Grand Island, NY, USA. Eti (purity > 98%) was acquired from TOKYO

CHEMICALINDUSTRY CO, LTD. Human neuroblastoma (SH-SY5Y) cells were purchased from F.D.C.C. Cobalt chloride ($CoCl_2$) and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl- 2-H-tetrazolium bromide (MTT) were purchased from Sigma Chemicals. OAG (30 μM) was purchased from Sigma (USA).

Cell Viability Assay by MTT

SH-SY5Y cells were cultured in DMEM high glucose medium supplemented with 10% FBS at 37 °C in the 95% humidified atmosphere with 5% CO_2. SH-SY5Y cells were seeded at an approximate density of 2×10^4 cells/well in 96-well culture plates for 24 h. Then the medium was replaced with different concentrations of $CoCl_2$ (0.2, 0.4, 0.6, 0.8, 1 mM) medium for 24 h. The next viability tests were carried out. SH-SY5Y cells were incubated with 100 μM Eti for 12 h, and then cells were incubated in 0.8 mM $CoCl_2$ for 24 h. Subsequently, 10 μL MTT (0.5 mg/mL) indicator was added and incubated for 4 h at 37 °C. Finally, MTT was removed and added 150 μL DMSO into each well. The absorbance was tested using a microplate reader at wavelength 578 nm.

Electrophysiological Experiments

For electrophysiological studies, cells were seeded in 35 mm culture dish at a density of $3–7 \times 10^4$ cells/mL and were cultured for 24 h. Then Eti solutions were added into the culture medium with 100 μM for 12 h. Finally, the culture medium was removed and 0.8 mM $CoCl_2$ was added for a further 24 h.

The cultured SH-SY5Y cells were divided into four groups as follows: (1) CON group, cells were cultured in 10% FBS medium. (2) $CoCl_2$ group, SH-SY5Y cells were cultured in 10% FBS medium with 0.8 mM $CoCl_2$. (3) Eti + $CoCl_2$ group, cells were cultured in 10% FBS medium with 100 μM Eti for 12 h, then replaced it with 0.8 mM $CoCl_2$ medium. (4) Eti + $CoCl_2$ + OAG group, 30 μM OAG, which activated TRPC channel currents, were added into Eti + $CoCl_2$ group. (5) Eti group (negative control group), cells were cultured in 10% FBS medium with 100 μM Eti for 12 h, then replaced it with 10% FBS medium.

SH-SY5Y cells were recorded using voltage clamp by the whole-cell configuration at room temperature (22–24°C). And experiments were carried out using EPC-10 patch-clamp amplifiers. Patch-pipettes with 7–9 MΩ resistance that filled with internal solution were used. For recording TRPC channel currents, the internal solution consisted of (mM): 130 CsCl, 130 L-aspartic acid, 2 $MgCl_2$, 0.3 $CaCl_2$, 10 HEPES, 10 EGTA and 3 ATP-Na at pH 7.3. And the external solution contained (mM):130 NaCl, 4 KCl, 1 $MgCl_2$, 10 HEPES, 10 glucose and 2 $CaCl_2$ at pH 7.3. The currents were measured by voltage ramps from − 100 to + 100 mV (200 ms) for holding potential of − 90 mV and were sampled at a rate of 4 kHz. It filtered at 2 kHz [24]. Recordings were discarded if the series resistance and leak current changed significantly and/or reached ≥ 30 MΩ or ≥ 350 pA, respectively.

Ca^{2+} Imaging

SH-SY5Y cells were seeded onto glass coverslips. Then the cells were washed three times with PBS and loaded with a final concentration of 5 μM Fluo-3/AM (Sigma, USA) at 37 °C for 30 min in dark. Then the fluorescence was observed at a laser wavelength of 488 nm, and wavelength of the emission fluorescence was 525 nm by confocal laser scanning microscopy (Olympus, Japan).

Immunofluorescence Staining

Cultured cells were grown on coverslips. Then cells were washed in PBS for three times. Subsequently, the cells were exposed to 4% paraformaldehyde for 30 min, They were then washed with PBS, following 10 min permeabilization in 0.5% Triton X-100 and blocked with 10% NGS for 2 h at room temperature. Where after, the cells were incubated with primary antibodies including anti-TRPC5 (1:1000, Abcam ab189262) and anti-HIF-1α (1:500 Abcam ab2185) antibody overnight at 4 °C. After washing with PBS for three times, they were incubated with the Alexa 488-conjugated goat anti-mouse IgG (1:1000, CA11008S; Invitrogen) and Alexa 594-conjugated goat anti-rabbit IgG (1:1000, A21235; Life Technologies). The fluorescent signals were detected under a fluorescence microscope (Olympus FV1000, Japan).

Western Blotting

After drug treatment, the SH-SY5Y cells were washed three times with PBS and were harvested lying with 100 μL ice-cold lysis buffer for 15 min (Cell signaling, Danvers, MA). Then the lysates were centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant contains total-proteins. Mitochondrial proteins were extracted using Tissue Mitochondrial Isolation Kit (Beyotime, C3606). Next, protein concentrations were determined using the BCA Protein Assay Kits according to the manufacturer's instructions (Beyotime Biotechnology, Haimen, China). Finally, the supernatant was mixed with 4× loading buffer (ratio is 3:1), and then boiled at 100 °C for 15 min.

Equal amounts of total-proteins were run on a 10–13% SDS-PAGE gel. After the separation on a polyacrylamide gel, the proteins were transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA).

After being blocked in Tris-buffered saline (TBS) including 0.1% Tween 20 and 5% fat-free dry milk for 1 h at room temperature, the PVDF membranes were incubated with primary antibodies (anti-HIF-1α (Abcam, ab2185) 1:500 dilution, anti-TRPC5 (Abcam, ab189262) 1:1000 dilution, anti-TRPC6 (Cell Signaling technology, #16716) 1:1000 dilution, anti-PARP (Cell Signaling technology, #9542) 1:1000 dilution, anti-caspase-3 (Cell Signaling technology, #9662) 1:1000 diltution, anti-caspase-9 (Abcam, ab202068) 1:1000 dilution, anti-CaMKII (Abcam, ab52476) 1:1000 dilution, anti-phospho-CaMKII (Abcam, ab32503) 1:1000 dilution, anti-cytochrome c (Abcam, ab110325) 1:1000 dilution, anti-ACTB (β-actin; 1:5000, Sangon, China), TOMM20 (Abcam, ab186734) 1:1000 dilution) diluted in blocking buffer overnight at 4 °C. Then the PVDF membranes were washed for 4 × 10 min with Tris-buffered saline/Tween 20 (TBST) and were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG (H + L), HRP conjugate (1:5000, Promega) or anti-mouse IgG (H + L) HRP conjugate (1:5000, Promega) for 1 h at a room temperature. After 4 × 10 min TBS washing, the blots were detected with a chemiluminescence detection kit (Pierce) and exposed to a X-ray film (Eastman Kodak, Rochester, NY). Finally, the density of the bands on Western blotting was carried out using Image J software (NIH, Bethesda, MD, USA).

Data Acquisition and Statistical Analysis

Whole-cell voltage-clamp recordings of SH-SY5Y cells (6–12 cells per group) were carried out at 37 °C. Our study used double blinding method for group assignment and outcome assessment. Data were acquired using an EPC10 amplifier (HEKA, Germany) connected to a computer and stored on a hard disk using pulse 8.52 software (HEKA, Germany), analyzed off-line using the pCLAMP 9.0 and Origin 9.1. All data were presented as mean ± SEM. Data were assessed by one-way analysis of variance (ANOVA) and Tukey's multiple comparison post-test using the SPSS (17.0) software. Throughout, $P < 0.05$ is regarded as significant.

Results

Eti-Pretreatment Improved the Damaged SH-SY5Y Cells that Induced by $CoCl_2$

In our experiments, MTT assay was used to examine the effect of different concentrations of $CoCl_2$ on viability of SH-SY5Y cells for 24 h. As shown in Fig. 1b, the cell viability gradually decreased from 0.2 to 1 mM, and when the concentration of $CoCl_2$ reached 0.8 mM, SH-SY5Y cell viability was 51%. Thus 0.8 mM was used for subsequent experiments. Then, in order to determine whether pre-Eti could inhibit $CoCl_2$-induced cell death, the MTT assay was used to perform on SH-SY5Y cells pre-incubated with Eti in DMEM high glucose medium supplemented with 10% FBS for 12 h followed by exposure to 0.8 mM $CoCl_2$. Moreover, Fig. 1c showed that pretreatment with 100 μM Eti could significantly improve the cell viability to 59%. In order to show that Eti did not exhibit cytotoxic effects in SH-SY5Y cells, the cells were treated with 100 μM Eti for 12 h. And there was no significant difference in cell viability compared with CON group.

Eti Pretreatment Attenuated Caspase-9 and Caspase-3 Activities Induced by $CoCl_2$ in SH-SY5Y Cells

Apoptosis is an important physiological process of cell death and mediates all sorts of biological events [25]. Some authors have reported that $CoCl_2$ could mimic the hypoxic/ischemic condition and induce apoptosis in various cells. Therefore, we speculated whether pretreatment-Eti can protect neurons by altering the apoptotic pathway.

Caspase activation is crucial mediators in the process of apoptosis. Therefore, the apoptosis-related proteins including the caspase family (caspase-9, caspase-3), PARP and Cyt-c were detected in our experiments (Figs. 2, 3). Compared with CON group, the expression levels of PARP, Cyt-c, cleaved-caspase-9, and cleaved-caspase-3 obviously increased in $CoCl_2$ group (Figs. 2a, 3a). The statistical results suggested that there were significant differences between CON and $CoCl_2$ group (Figs. 2b–d, 3b). In addition, the expression of Cyt-c in mitochondria was examined by Western blot assay in Fig. 4a. Compared with CON group mitochondrial Cyt-c protein was decreased in $CoCl_2$ group. But mitochondrial Cyt-c protein was increased in Eti + $CoCl_2$ group compared with $CoCl_2$ group (Fig. 4b). It suggested that Cyt-c in the mitochondria was released into the cytoplasm, activating the apoptotic pathway. The Western blot results showed that the expressions of apoptotic proteins were significantly decreased in Eti + $CoCl_2$ group (Figs. 2a, 3a). And the statistical results are proved consistent (Figs. 2b–d, 3b). These results indicated that pre-Eti treatments could protect cells from apoptosis caused by $CoCl_2$. Moreover, the ratio of LC3-II/LC3-I and the expression of Atg7 protein were obviously increased in $CoCl_2$ group (Figs. s1 and s2). It suggested that $CoCl_2$ may induce autophagy in SH-SY5Y cells.

As reported in the literature, the expression of hypoxia inducible factor 1 (HIF-1) was increased by $CoCl_2$-induced hypoxia injury, but also the $CoCl_2$ activated apoptosis. Consequently, we hypothesized that pre-Eti treatment protected SH-SY5Y cells from $CoCl_2$-induced apoptosis because of the inhibition of HIF-1α. Therefore, we conducted the following up experiment described below.

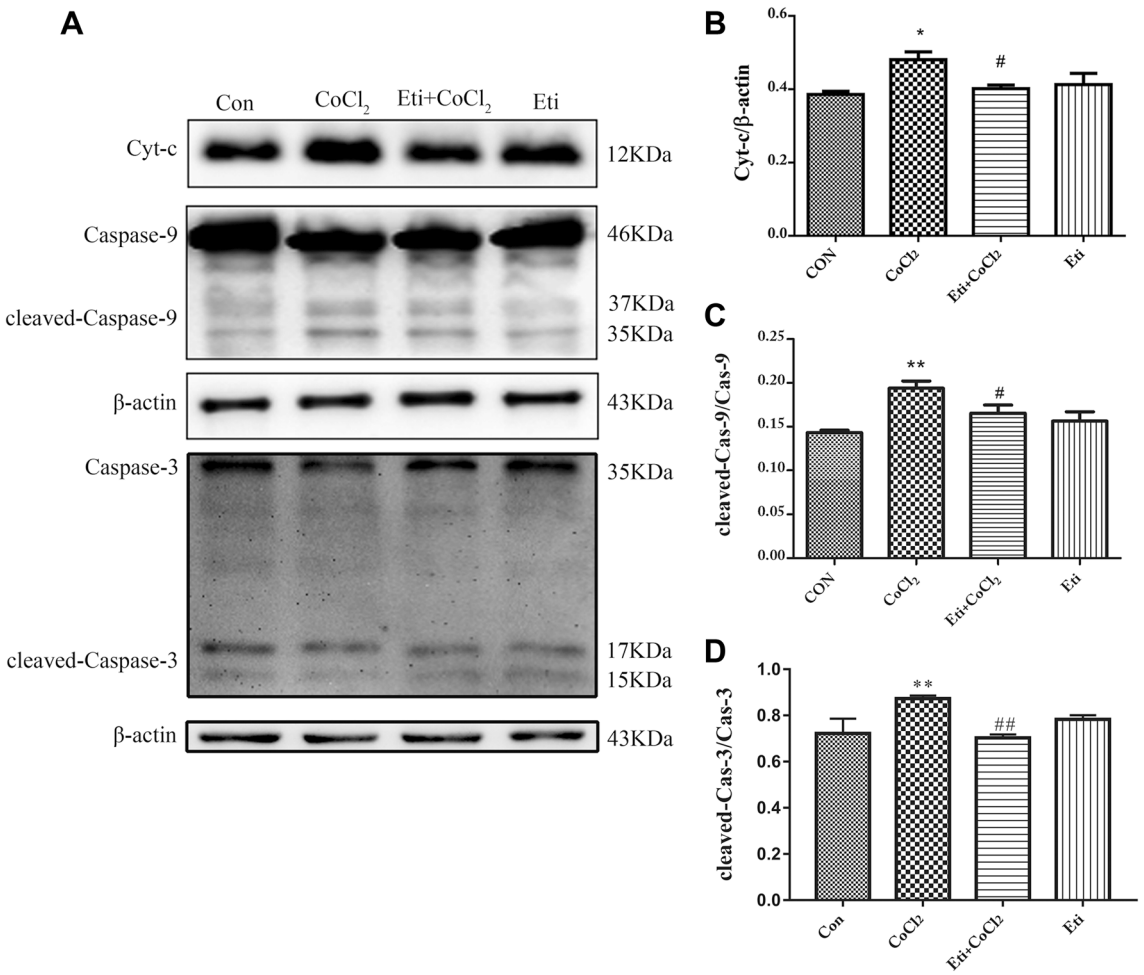

Fig. 2 Effects of Eti on apoptosis. **a** Expressions of cytochrome c (Cyt-c), caspase-9 (Cas-9), cleaved-caspase-9 (cleaved-Cas-9), caspase-3 (Cas-3) and cleaved-caspase-3 (cleaved-Cas-3) proteins tested by Western blot assay. **b–d** the ratio of Cyt-c, caspase-9, cleaved-caspase-9, caspase-3 and cleaved-caspase-3 proteins. Results of statistical analysis by Image J software. All data were presented as the means ± SEM, n = 3. *p < 0.05 comparison between CON group and $CoCl_2$ group. **p < 0.01 comparison between CON and $CoCl_2$ group; #p < 0.05 and ##p < 0.01 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

Eti-Pretreatment Reduced the Expression of HIF-1α in SH-SY5Y Cells

The key regulator of the hypoxia response is the HIF-1. HIF-1 is a complex heterodimer including a variety of subunits (HIF-1α and HIF-1β) [26], of which HIF-1α causes hypoxia. $CoCl_2$ has been reported to induce hypoxia response and apoptosis by the HIF-1α. Several studies have also indicated that $CoCl_2$ can determine the stability of HIF-1α regulatory subunits [27–29]. Thus HIF-1α plays a crucial role in the hypoxia response. In our study, in order to investigate whether or not HIF-1α is correlated to the development of hypoxia-response induced by $CoCl_2$ within the cultured SH-SY5Y cells, we detected the expression of HIF-1α protein through Western blot assay and immunofluorescence (Fig. 5). The results of Western blot assay (Fig. 5a) suggested that the expression of HIF-1α increased in SH-SY5Y cells after treated with 0.8 mM $CoCl_2$ for 24 h. In a contrast, HIF-1α protein was decreased in Eti + $CoCl_2$ group. And the results of immunofluorescence are consistent with the results of Western blot assay. The red fluorescence(HIF-1α) intensity in $CoCl_2$ group is the largest. From these results, the expression of HIF-1α was significantly increased in the $CoCl_2$ group, whereas HIF-1α was significantly decreased in Eti + $CoCl_2$ group. The above results of Figs. 2 and 5 are also similar to the other discussions in literatures, which suggested that HIF-1α involved in hypoxia response and induced apoptosis [30, 31].

The involvement of HIF-1α in hypoxia response suggested that $CoCl_2$ could promote cell apoptosis by inducing HIF-1α expression. And the Eti pretreatment reduced the expression of HIF-1α in SH-SY5Y cells by playing a protective role in the cells.

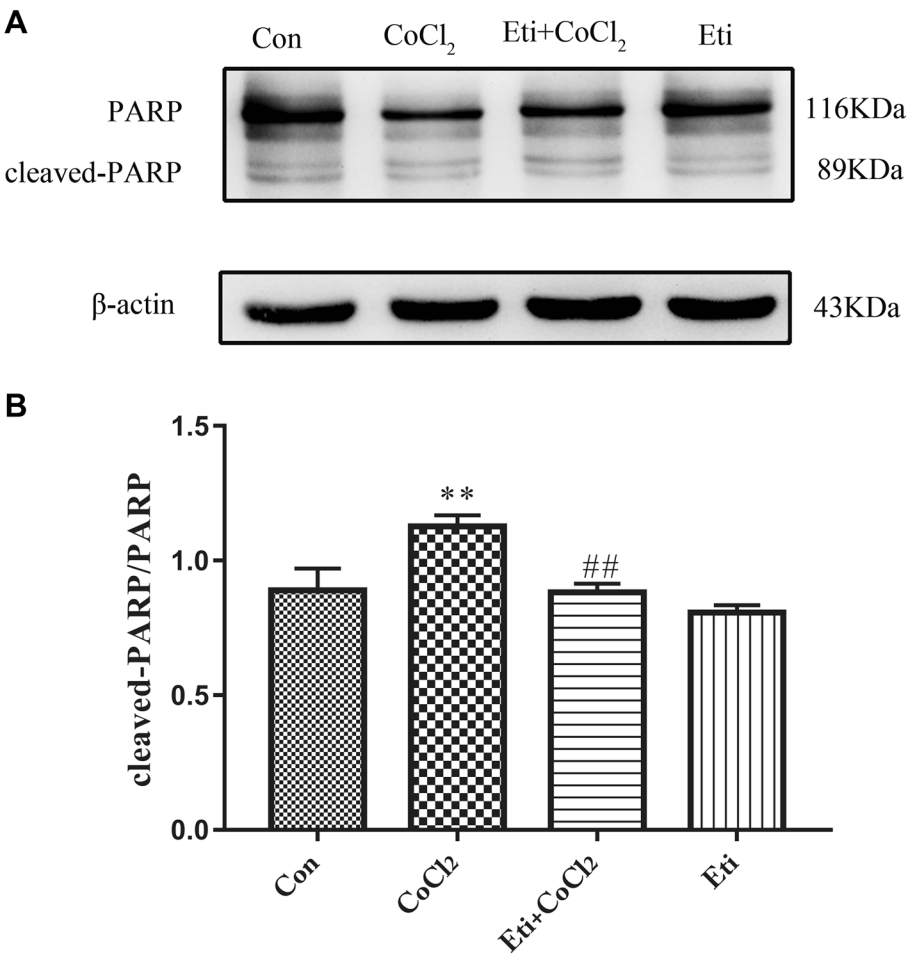

Fig. 3 Results of Western blot assay for expressions of PARP and cleaved-PARP protein. **a** Schematic diagram of immunoblotting of PARP protein. **b** The statistical result of PARP protein. **p < 0.01 comparison between CON and $CoCl_2$ group; ##p < 0.01 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

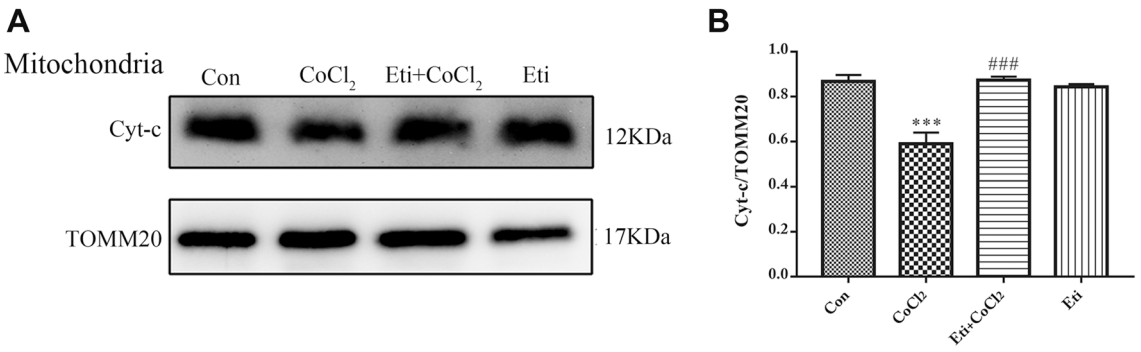

Fig. 4 Schematic diagram of cytochrome c(Cyt-c) expression in mitochondria. **a** The expression of Cyt-c in mitochondria tested by Western blot assay. **b** Statistical results of each group about Cyt-c in mitochondria. ***p < 0.001 comparison between CON and $CoCl_2$ group; ###p < 0.001 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

Eti-Pretreatment Inhibited the Activity of Ca^{2+}-CaMK-II Signals

Ca^{2+} plays an important role in SH-SY5Y cells. And the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is associated to the cell apoptosis, as the intracellular second messenger [32]. Changes in cytoplasmic $[Ca^{2+}]_i$ can affect different calcium-coupled processes, such as secretion, contraction, photoreception, protein activation, fertilization, proliferation, and apoptosis [33]. And CaMK-II is a ubiquitous serine/threonine protein kinase that was activated by Ca^{2+} and calmodulin (CaM) and has been implicated in cell

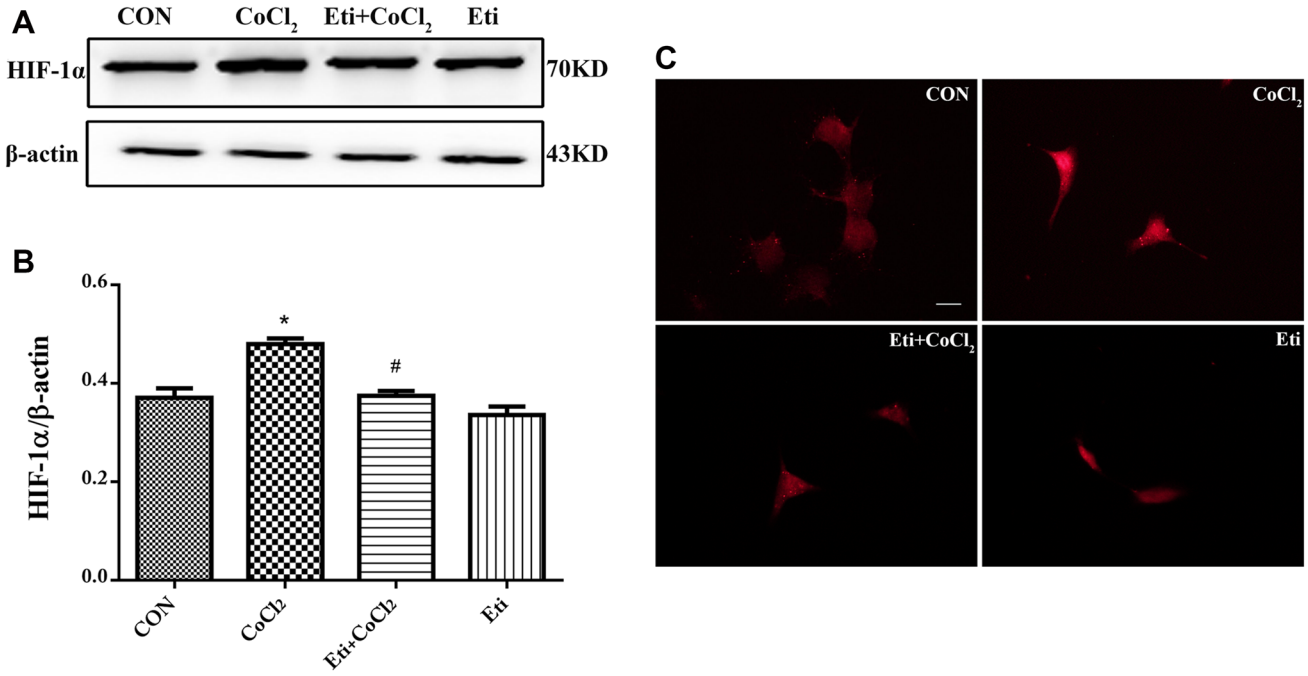

Fig. 5 The expression of HIF-1α protein analyzed by Western blot assay and immunofluorescence staining. Scale bar 20 μm in **c. a, b** Representative cropped Western blots and statistical analysis by Image J software. **c** Immunofluorescence staining. All data were expressed as mean ± SEM, n = 3. *p < 0.05 comparison between CON group and $CoCl_2$ group; #p < 0.05 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

cycle control [34, 35]. Therefore, $[Ca^{2+}]_i$ was measured in this work by using the Ca^{2+}-sensitive dye named fluo-3/AM in SH-SY5Y cells when exposed to $CoCl_2$ and Eti-pretreatment. The results of Ca^{2+} fluorescence (Fig. 6a) showed that a higher fluorescent intensity was observed in $CoCl_2$ group (Fig. 6c) in comparison to the CON group (Fig. 6a). While the fluorescent intensity was significantly reduced in Eti + $CoCl_2$ group compared with the $CoCl_2$ group (Fig. 6c).

As a pivotal second messenger, $[Ca^{2+}]_i$ affects a series of intracellular cascade reactions including Ca^{2+}-CaMK-II signaling pathways. Therefore, CaMK-II and phosphorylated CaMK-II (p-CaMK-II) protein expression were tested, and the results are as shown in Fig. 6b. The outcomes demonstrated that chronic hypoxic mediated by $CoCl_2$ could induce Ca^{2+} flux into cell and activated phosphorylation of CaMK-II. It also showed that compared with $CoCl_2$ group, the enhanced expression of pho-CaMK-II was blocked by Eti-pretreatment (Fig. 6d). Together, $CoCl_2$-mediated Ca^{2+}-CaMK-II signaling was inhibited by Eti-preteratment. Since the up-regulation of Ca^{2+}-CaMK-II signaling pathway can activate the apoptosis, we speculated that Eti.-pretreatment down-regulated the Ca^{2+}-CaMK-II signaling pathway by decreasing the $[Ca^{2+}]_i$. After this, we explored how the Eti-pretreatment reduced the $[Ca^{2+}]_i$ in next section.

Eti-Pretreatment Decreased $[Ca^{2+}]_i$ by Inhibiting the Activation of TRPC Channels

An important reason for the increase of $[Ca^{2+}]_i$ is the activation of calcium-related channels in the cytomembrane. TRPC channels are non-selective Ca^{2+} channels, which mainly mediate calcium ions flux into cells. And studies have showed that the expression of TRPC was increased by up-regulation HIF-1α in cultured neonatal rat cardiac myocytes [36]. Therefore we detected the relevant TRPC channel protein and HIF-1α protein. Based on the above experimental results, we found that $CoCl_2$ improved the expression of HIF-1α, while the pretreatment-Eti reversed this result (Fig. 5).

At the same time we also found that the expression of TRPC5 channel protein was improved in $CoCl_2$ group, but the expression of TRPC6 channel protein didn't change (Fig. 7). Then the expressions of TRPC5 and HIF-1α were also detected by immunofluorescence staining (Fig. 8), and the results were consistent with that of Western blot assay (Fig. 7). And these results showed that the expression of TRPC5 increased/decreased with the increased/decreased HIF-1α in the $CoCl_2$-group and Eti + $CoCl_2$ group. Therefore, we speculated that $CoCl_2$ improved the expression of TRPC5 channel protein by increasing HIF-1α.

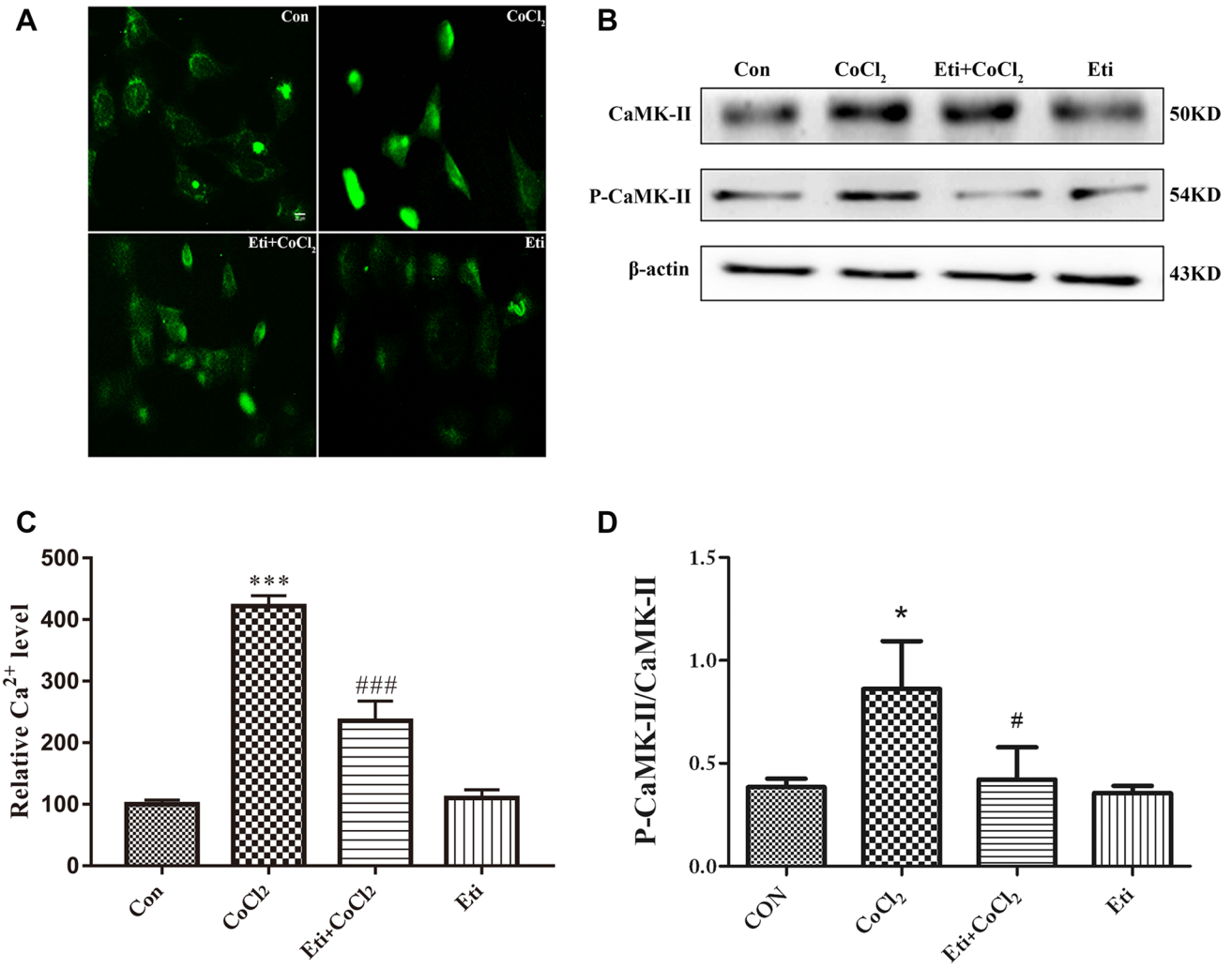

Fig. 6 Fluorescent intensity of intracellular Ca^{2+} in SH-SY5Y cells. **a** Fluorescent intensity of intracellular Ca^{2+} in each group including 0.8 mM $CoCl_2$ alone, 100 mM Eti + 0.8 mM $CoCl_2$, 100 mM Eti alone and CON group. And Scale bar 20 μm in **a**. **b** Results of CaMK-II and p-CaMK-II protein expression measured by Western blot assay and **c, d** statistical analysis of relative intracellular Ca^{2+} level and the ratio of p-CaMK-II and CaMK-II by Image J software and SPSS. Data were presented as the means ± SEM, n = 3. *p < 0.05 comparison between CON group and $CoCl_2$ group; ***p < 0.001 comparison between CON group and $CoCl_2$ group; #p < 0.05 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group; ###p < 0.001 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

Based on previous experimental results, we found that treatment of $CoCl_2$ could increase the expression of TRPC5 channel proteins. Thus, to further evaluate the effects of $CoCl_2$ and Eti + $CoCl_2$ on TRPC channels, conventional whole-cell recording was used to measure the TRPC current density (Fig. 9). Recordings were performed at a holding potential of − 90 mV, TRPC currents were activated using a voltage-clamp, ramp protocol (1 mV/ms, from − 100 to 100 mV, over 200 ms) every 5 s. The I–V curves in Fig. 9a showed that both the inward and the outward currents were substantially increased in different groups. In these results, we observed a significant increase in current density in $CoCl_2$ group compared with that of CON group, while no significant change was found in Eti + $CoCl_2$ group. Then we measured the TRPC current density in presence of OAG (30 μM), which activated TRPC channel currents. Compared with $CoCl_2$ group, we observed an obviously increase in Eti + $CoCl_2$ + OAG group (Fig. 9b). These results indicated that Eti not only decreased the expressions of TRPC5 channel proteins, but also inhibited the activity of TRPC channel.

Furthermore, TRPC channels play an essential role in regulating calcium homeostasis of cell signaling. The results of calcium imaging (Fig. 6a) was consistent with TRPC channels current. Together with the above results, we proposed that pretreatment-Eti reduced the expressions of TRPC5 channel proteins and possibly changed the activation of TRPC channels in chronic hypoxic condition, which

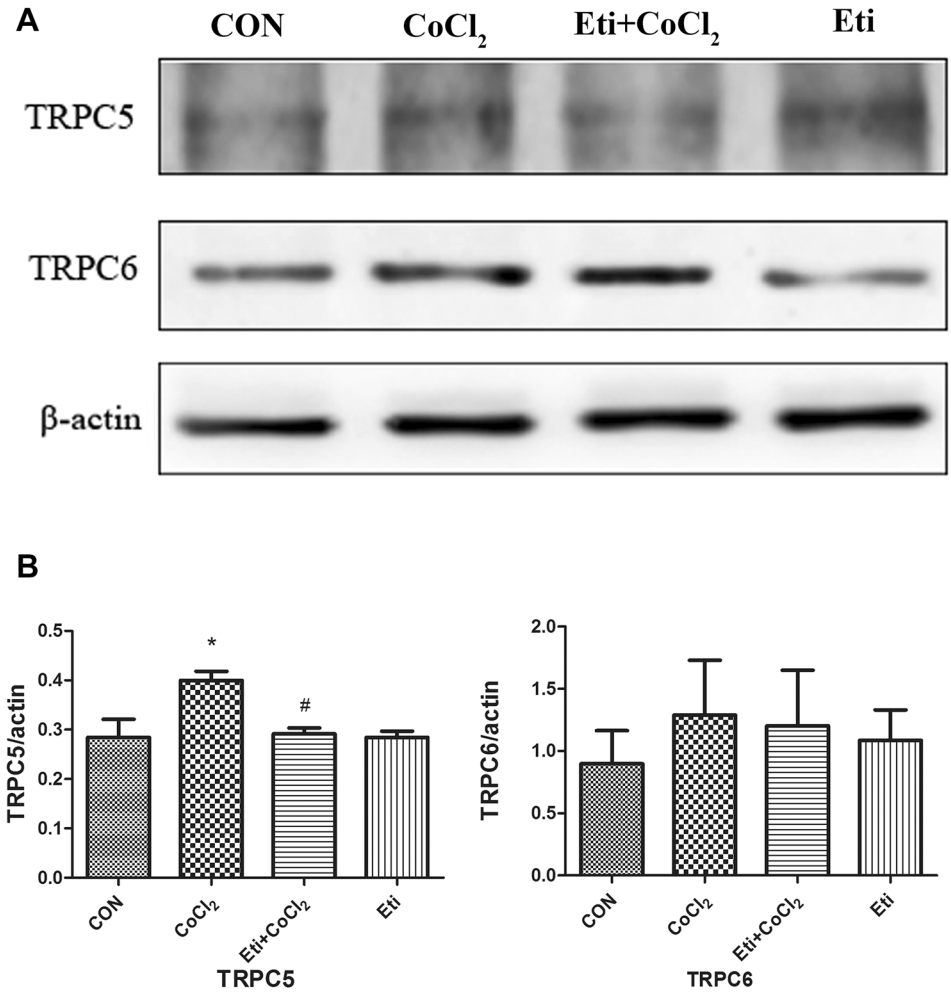

Fig. 7 TRPC5 and TRPC6 protein expressions analyzed by Western blot assay. **a** Western blots of TRPC5 and TRPC6 proteins. **b** Statistical analysis of TRPC5 and TRPC6 protein expression by Image J software. All data were expressed as mean ± SEM, n = 3. *p < 0.05 comparison between CON group and $CoCl_2$ group; #p < 0.05 comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

in turn affected the flow of Ca^{2+} into the cells, thereby affecting apoptosis.

Discussion

Usually, hypoxia can lead cells to apoptosis pathway, and apoptosis of neurons may result in some neurodegenerative diseases, such as vascular dementia, Alzheimer's disease, Parkinson's disease, epilepsy, etc. Therefore, it is important to find ways to avoid neurons suffering from anoxia injury.

Previous findings from this laboratory have showed that Eti not only rescued PC12 cells from excitability toxicity, but also improved the synaptic transmission and inhibited oxidative stress and apoptosis by regulating the concentration of intracellular Ca^{2+} [8, 9]. The objective of our study was to clarify how the Eti improved chronic hypoxic damage in SH-SY5Y cells, as a new potential neuronal protective drug. Building on the purpose, we designed a series of experiments, and the results achieved elucidated the mechanism of Eti, which regulated apoptosis by TRPC channels.

$CoCl_2$ is characterized by a hypoxia-mimetic agent in this study, a physiological stress method, which can lead to oxidative stress, causing the damages of proteins, lipids and DNA [37]. Chronic hypoxic is one of the most common pathogenic factors causing all kinds of cell death through apoptosis in vitro, such as in rat hepatoma cell line 7316A, human fibroblast cell line GM701, murine pro-B cell line BAF3, human lymphoid cell line SKW6.4, and rat pheochromocytoma cell line PC12 [38, 39]. And neuronal death may occur via necrosis or apoptosis in the model of cerebral hypoxia–ischemia in vivo [40]. Moreover, some studies have showed that $CoCl_2$ can be used to mimic the hypoxia/ischemic condition because it increases ROS and enhances the HIF-1α expression responsible for hypoxia-induced cell death in a variety of cells [19, 23, 41, 42]. In order to determine whether $CoCl_2$ can cause chronic hypoxia, the expression of HIF-1α protein was detected by Western blot analysis as well as immunofluorescence in SH-SY5Y cells. Firstly, the results of cell viability assay by MTT showed that with the increased $CoCl_2$ concentrations, the viability of SH-SY5Y cells decreased gradually (Fig. 1b). Secondly, the

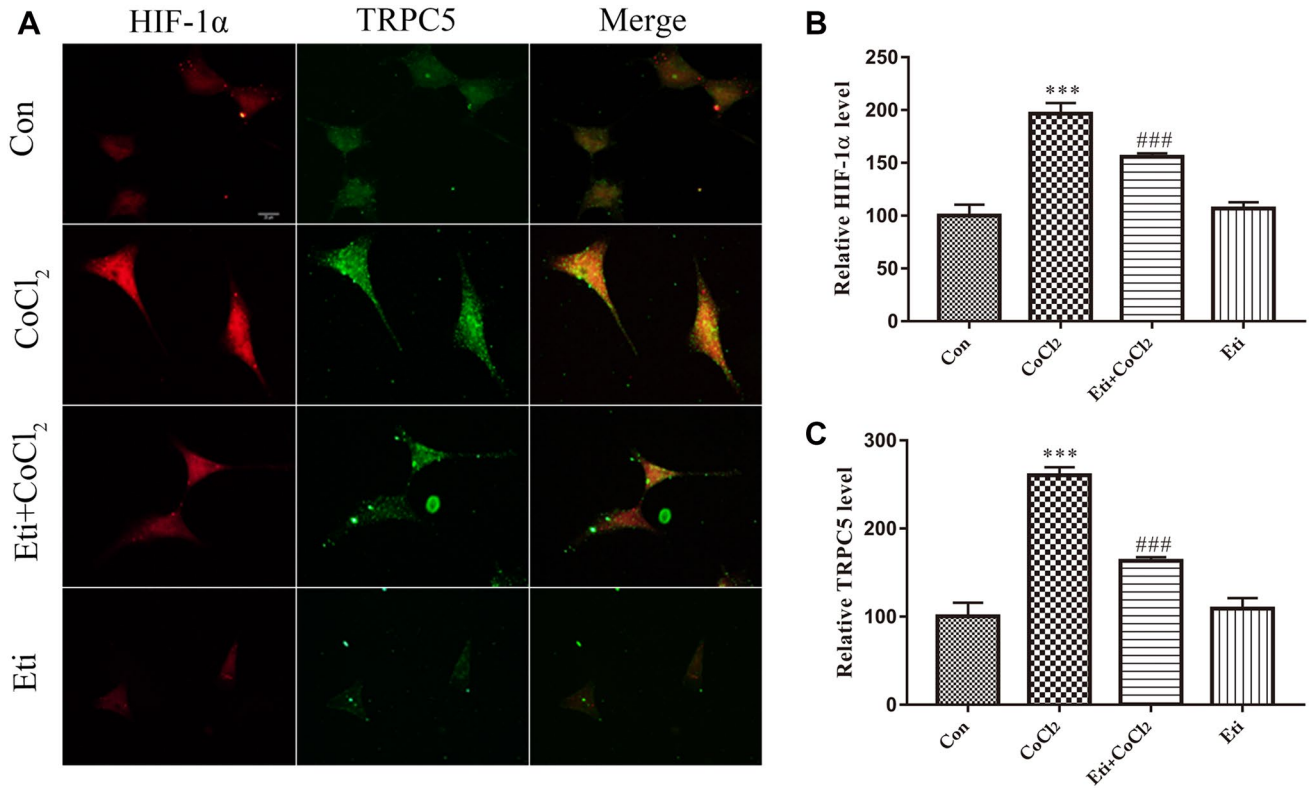

Fig. 8 Immunofluorescence staining of HIF-1α and TRPC5. **a** HIF-1α was red fluorescence, and TRPC5 was green fluorescence. Yellow fluorescence was displayed when HIF-1α overlaps with TRPC5. Red fluorescence and green fluorescence were highly expressed in the $CoCl_2$ group, indicating an increase in HIF-1α and TRPC5 expression. In Eti + $CoCl_2$ group, the fluorescence intensities of HIF-1α and TRPC5 were significantly decreased. Scale bar 20 μm. **b** Statistical analysis of HIF-1α and TRPC5 fluorescence values. ***$p < 0.001$ comparison between CON group and $CoCl_2$ group; ###$p < 0.001$ comparison between $CoCl_2$ group and Eti + $CoCl_2$ group

results of immunoblotting and immunofluorescence showed that the expression of HIF-1α protein was upregulated in $CoCl_2$ group (Fig. 5). These confirmed that $CoCl_2$ indeed induced a model of chronic hypoxia in SH-SY5Y cells in this study. Moreover, pretreatment-Eti could significantly rescue the decreased cell viability and the expression of HIF-1α protein (Figs. 1, 5). In order to explore the mechanism of how the Eti pretreatment improved the viability of SH-SY5Y cells, further investigation was carried out.

The expressions of proteins, which was related to apoptosis such as Cyt-c protein, caspase-9 and -3 protein and PARP protein were examined. Previous study reported that $CoCl_2$ can induce mitochondria-apoptosis in mES cells, and caspase-3 can play a key role in this program [43]. With a similar approaching methodology of Christophe et al. [44], our research demonstrated that SH-SY5Y cells exposed to $CoCl_2$ resulted in a classical apoptosis. Earnshaw et al. and Bal-Price and Brown also reported that caspase-3 was a crucial protease in both mitochondria and death receptor-dependent pathways, besides it played a vital role in free radical- mediated apoptosis [45, 46]. Consequently, Jin-Ha Lee et al. revealed that $CoCl_2$ enhanced caspase-9 and -3 activities in mES cells. Furthermore, $CoCl_2$ increased the Cyt-c released from the mitochondria accompanied by the loss of $\Delta\Psi_m$ and upregulated VDAC, which was one of the channel of Cyt-c release in mES cells [43, 47]. These articles hinted that apoptosis could be induced by $CoCl_2$-mediated chronic hypoxia through mitochondria and death receptor- mediated pathway in SH-SY5Y cells. In this study, the results indicated that $CoCl_2$ mediated apoptosis in SH-SY5Y cells through increasing the release of Cyt-c and the expression of cleaved-caspase-9 and cleaved-caspase-3 and cleaved-PARP (Figs. 2–4). And pretreatment-Eti could significantly reduce the expressions of these proteins. Taken together, pre pretreatment-Eti could dramatically improve apoptosis by inhibiting mitochondria-dependent apoptosis signaling pathways in SH-SY5Y cells.

Ca^{2+} is an important second messenger playing an essential role in the regulation of many cellular processes, which elicits dynamic changes and regulates biochemical responses [48, 49]. And the accumulation of intracellular Ca^{2+} can stimulate cell apoptosis. Our results indicated that Eti could reduce $[Ca^{2+}]_i$ and the expression of phosphorylation-CaMK-II protein, thereby improve SH-SY5Y cell hypoxia

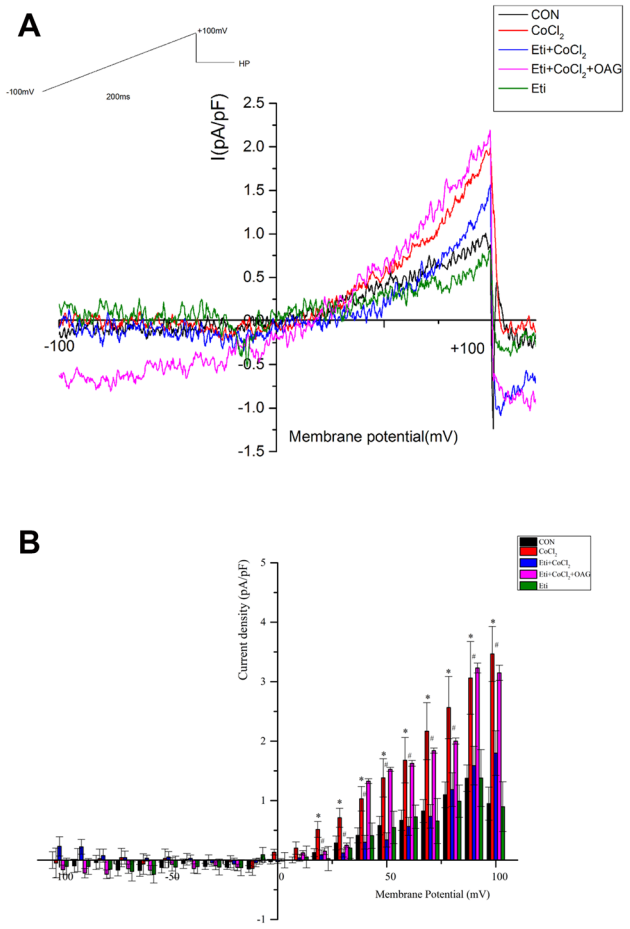

Fig. 9 The characteristic of TRPC currents in SH-SY5Y cells. Examples of membrane current recorded from SH-SY5Y cells. CON group and $CoCl_2$ group (n = 10, respectively), Eti + $CoCl_2$ group (n = 8), Eti + OAG group (n = 7). **a** Diagram of the current density of TRPC channels. **b** Statistical analysis of current density of TRPC channels

injury. Based on above, we could continue to explore how Ca^{2+} flows into cells.

With expansion on these findings, further work was performed to determine whether the Eti reflected Ca^{2+} channel activation. In a previous report, it was assumed that TRPC3 and six as the cation channels allowed Ca^{2+} influx to trigger cell death [50]. Moreover, Chu et al. reported that HIF-1α played an important role in mild hypoxia-induced cardiomyocyte hypertrophy, and it up-regulated TRPC3 and six expressions, enhanced Ca^{2+}-calcineurin signals by activating TRPCs channel currents [51]. In this study, we, for the first time, found that only the expression of TRPC5 protein was affected, whereas the expression of TRPC6 protein did not change in SH-SY5Y cells (Fig. 7). Based on this, we speculated that on the one hand hypoxia was induced by different ways, on the other hand the cell line was different. These experimental results indicated a crucial role of TRPC for regulating intracellular Ca^{2+} homeostasis. These results also proved that the TRPC5 played an important role in anti-apoptosis. It suggests that TRPC5 channel may target the location of Eti-effect in injured SH-SY5Y cells. Even though TRPC6 channel proteins are not affected, we wouldn't rule out the effect on the activity of TRPC6 channel.

Combined with previous studies of our laboratory, pre-Eti treatment can accurately play a neuroprotective effect through various pathways. Moreover, this study can clearly indicate pre-Eti treatment can regulate the TRPC channel family to protect neurons from chronic hypoxic damage for the first time. For the further research, we will clarify the role of Eti-regulated HIF-1α—TRPC pathway in neuroprotection.

Conclusion

For the first time, our research result proved that Eti can be served as a neuroprotective drug, and protect cells from chronic hypoxia injury caused by $CoCl_2$ as shown in Fig. 10. On the one hand, results showed that pre-Eti treatment can reduce TRPC5 channel protein expression so that the intracellular calcium ion concentration was reduced to avoid calcium overloading. Subsequently, the pre-Eti treatment inhibited the CaMK-II phosphorylating, caspase-9 and caspase-3 activation. On the other hand, pre-Eti treatment can reduce the expression of intracellular HIF-1α protein and present anti-apoptosis properties. Hence, this research suggests that Eti serves as a new type of neuroprotective drug to protect cells from damage.

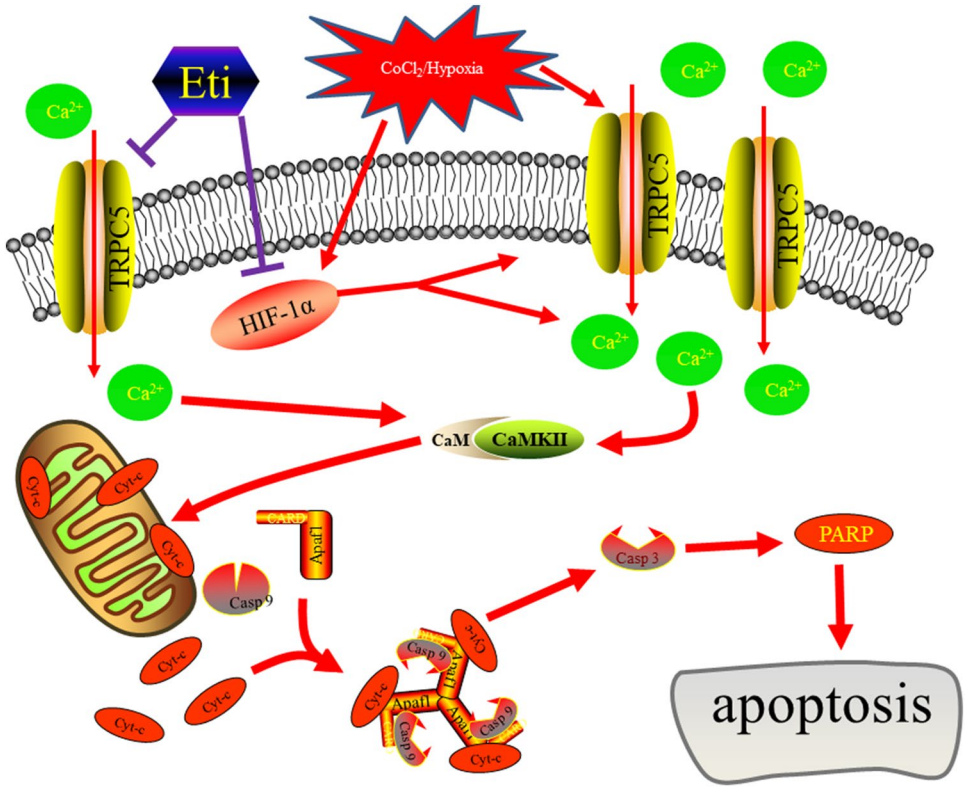

Fig. 10 Schematic figure illustrates the mechanism of Eti protection of SH-SY5Y cells against $CoCl_2$-induced chronic hypoxic damage. The red arrow indicates activation, and the violet line indicates suppression. $CoCl_2$ causes an increase of intracellular Ca^{2+}([Ca^{2+}]i) and HIF-1α protein in SH-SY5Y cells, and activates the release of cytochrome c (Cyt-c) from the mitochondria into the cytosol, activating apoptotic pathway. The pretreatment of Eti changes the activity and expression of the TRPC5 channels, thereby preventing intracellular calcium overload, which decreases the activation of apoptotic proteins to protect cells from death. Abbreviations: Etidronate(Eti), transient receptor potential channel(TRPC), calmodulin(CaM), calmodulin kinase II(CaMKII), hypoxia-inducible factor 1α(HIF-1α), cytochrome c (Cyt-c), caspase-9(Cas-9), caspase-3(Cas-3)

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Compliance with Ethical Standards

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

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