Hypoxia-inducible factor-2 α but not hypoxia-inducible factor-1 α increases iron traffic in astrocytes

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Abstract

Background: Disrupted iron homeostasis in the substantia nigra (SN) is an important mechanism in Parkinson's disease (PD). In our previous studies using cultured neurons, we showed that 6-hydroxydopamine (6-OHDA) treatment increased the expression of divalent metal transporter-1 (DMT1) gating iron influx and decreased the expression of ferroportin 1 (FPN1) gating iron efflux, leading to increased iron deposition. In astrocytes, 6-OHDA increased both DMT1 and FPN1 expressions and iron traffic. However, the underlying mechanisms mediating these processes remain elusive. Hypoxia-inducible factors (HIFs) are important regulators of iron homeostasis.

Methods: Using western blots, we observed HIFs, DMT1 and FPN1 expressions in primary cultured astrocytes and ventral mesencephalic (VM) treated with 6-OHDA, inhibitors of HIF-1 α and HIF-2 α , protein kinase C (PKC) inhibitor and PKC activator, radical scavenger and inducible NO synthase (iNOS) inhibitor. The ferrous iron traffic of astrocytes was determined by measuring the quenching or reversing of calcein fluorescence.

Results: Using primary rat cell cultures, we observed that 6-OHDA treatment significantly increased the expressions of HIF-1 α and HIF-2 α in cultured astrocytes but not in VM neurons. Moreover, we observed that HIF-2 α inhibitor, but not HIF-1 α inhibitor reversed the 6-OHDA-induced upregulation of DMT1 and FPN1 and ferrous iron traffic. Blocking protein kinase C (PKC) pathway by bisindolylmaleimide I hydrochloride (Bisl) or N-acetyl-L-cysteine (NAC) or N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME) inhibits Phorbol 12-myristate 13-acetate (PMA) or 6-OHDA induced activation of HIF-2 α , whereas activating by PMA or 6-OHDA was sufficient to activate HIF-2 α . Notably, blocking of PKC δ phosphorylation by NAC, or L-NAME inhibits 6-OHDA induced activation of HIF-2 α .

Conclusion: Our data indicate that astrocytic HIF-2 α , but not HIF-1 α , may be an important regulator of DMT1 and FPN1 expressions by activating the PKC-dependent pathway.

Background

Parkinson's disease (PD) is a neurodegenerative disorder characterized in its late phase by the sustained loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Clinically PD is characterized by bradykinesia, rigidity, rest tremor and postural instability. Although multiple factors such as genetic mutation, environmental factors, and aging might be involved in PD pathogenesis, its underlying mechanisms have not been revealed yet. In both animal PD models and PD patients, selective deposition of iron in the substantia nigra (SN) has been observed, while no iron content increases have been observed in other brain regions¹. Most current studies using PD models focused on iron deposition in dopaminergic neurons. However, a variety of gliocytes also plays important roles in cellular iron homeostasis². For example, astrocytes were found to have high iron tolerance^{3,4}. During iron overload, neurons, microglia and oligodendrocytes all have iron deposit, but astrocytes does not have iron deposit in the same situation⁴⁻⁶. In our previous studies⁷⁻¹⁰, we reported that in neurons, 6-hydroxydopamine (6-

OHDA) activate iron regulatory protein 1 (IRP1), increased the expression of divalent metal transporter-1 (DMT1) and decreased the expression of ferroportin 1 (FPN1), led to increased iron influx and decreased iron efflux. In astrocytes, 6-OHDA increased both DMT1 and FPN1 expressions while keeping the expression of IRP1 unchanged, therefore increased iron traffic^{11, 12}. Taken all those evidence together, the mechanisms of iron metabolism regulations in neurons and astrocytes were different, but the detailed mechanism underlying iron metabolism in astrocytes remain elusive.

Astrocytes play an essential role in blood-brain barrier (BBB) formation, regulating brain iron homeostasis and preventing damage from iron-mediated oxidative stress¹³. For example, astrocytes end feet formations the BBB which restricts the levels of the majority of metal ions in the central nervous system (CNS)¹⁴. Astrocytes also regulates iron transport from themselves to other brain cells^{6, 15}. In addition, astrocytes expressed almost all the iron transporters and distinct profiles of iron metabolism proteins, including DMT1 which transports iron into the cells and FPN1 which transports iron out¹⁶⁻¹⁹. As one of the components of BBB, it is reasonable to hypothesize that astrocytes may regulate iron metabolism in a similar way with small intestine epithelium cell. In particular, astrocytes do not have much ferritin and iron, indicating that these cells provide little iron storage^{16, 20-22}. Moreover, hypoxia-inducible factor-2 α (HIF-2 α) is the key transcription factor responsible for increasing iron absorption in the intestine epithelium cell. This is mediated by the binding of HIF-2 α to hypoxic response elements (HREs) on promoters for DMT1 and FPN1²³. Therefore, we reason that HIF-2 α may also regulates the iron metabolism in astrocytes.

Hypoxia-inducible factors (HIFs) are involved in regulating iron homeostasis and are heterodimers consisting of two helix-loop-helix-containing subunits: a oxygen-responsive regulatory subunit and a constitutively expressed HIF-1 β , also known as the arylhydrocarbon receptor nuclear translocator (ARNT),²⁴. Three regulatory HIF subunits have been characterized: HIF-1 α ²⁴, HIF-2 α ²⁵ and HIF-3 α ²⁶. HIF-1 α is expressed ubiquitously²⁷, whereas HIF-2 α expression appears to be restricted to certain tissues²⁸. The expression patterns and functional properties of HIF-3 α remain to be elucidated²⁹. Binding of HIF-2 α to the promoter region of the HREs of DMT1 and FPN1 increases their expression³⁰. In mice, conditional knockouts of HIF-2 α in the intestine epithelial cells significant decreases the levels of DMT1 and FPN1 mRNA in duodenal, effects that were not compensated for by HIF-1 α . Therefore, it appears that HIF-2 α , but not HIF-1 α , regulates expression of DMT1 and FPN1 in the intestine³¹. Although in peripheral tissues iron homeostasis seems to occur via HIF-2 α but not HIF-1 α , studies have reported that HIF-1 α does regulate the expression of DMT1 and FPN1 in brain^{32, 33}. Therefore it is unclear whether HIF-1 α or HIF-2 α plays a more critical role in astrocytic iron traffic

Protein kinase C (PKC) modulates the HIF pathway in the human cervical cancer cell line HeLa and one subtype PKC δ plays an important role in regulating HIFs. Activation of PKC upregulates the expressions of HIF-1 α / HIF-2 α in HeLa, PC3, or HT1080 cancer cell lines^{34, 35}, ROS/RNS (mainly H₂O₂ and NO) functions as second messengers to regulate the function of proteins involved in the PKC pathway, and consequently activates HIFs. Moreover, PKC α and PKC δ regulate HIFs aggregation in these cell lines³⁵⁻

³⁸. In our previous studies, we found that like PKC α did not involved in the neurotoxicity of 6-OHDA, whereas PKC δ and PKC ζ were activated by 6-OHDA in DA neurons ³⁹. Moreover, in peripheral tissues, the activation of PKC and the regulation of HIFs under oxidative stress is mainly through the activation of PKC δ ³⁵. Whether PKC δ plays an critical role in the regulation of HIFs in astrocytes remain to be proven.

Currently, it is unclear whether astrocytes contribute to iron metabolism, and whether the iron which accumulates in SN neurons in PD comes from astrocytes. Furthermore, the role of HIFs in iron metabolism in astrocytes and the roles of the PKC signaling pathway and ROS/RNS in regulating HIFs remain to be elucidated. Therefore in the present study, we used treatment with 6-OHDA to investigate the role of HIFs in astrocytic iron metabolism and iron traffic. We hypothesized that 6-OHDA activates HIFs via PKC activation, and that 6-OHDA induces ROS and NO to activate HIFs, thereby influencing iron metabolism in astrocytes.

Methods

Materials

All procedures were performed in accordance with the International Guiding Principles for Biomedical Research Involving Animals. We purchased 6-OHDA, L-ascorbic acid, ferrous sulfate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$), deferoxamine (DFO), dimethyl sulfoxide (DMSO), phorbol 12-myristate-13-acetate (PMA), bisindolylmaleimide I hydrochloride (BisI), N-acetyl-L-cysteine (NAC), N ω -Nitro-L-arginine methyl ester hydrochloride (L-NAME), and primary antibodies against HIF-1 α and FPN1 from Sigma Chemical Co. (St Louis, MO, USA). The HIF-1 α inhibitor BAY 87-2243 was obtained from Selleckchem (Houston, TX, USA), and HIF-2 α translation inhibitor was acquired from Millipore (Billerica, MA, USA). The primary DMT1 antibody was purchased from OriGene Technologies, Inc. (Rockville, MD, USA). The primary HIF-2 α antibody was from Novus Biologicals (Littleton, CO, USA) and rabbit anti- β -actin was from Bioss (Beijing, China). We purchased Calcein-AM from Molecular Probes Inc. (Carlsbad, CA, USA). Dubecco's modified Eagle's medium (DMEM)/F12 and fetal bovine serum (FBS) were sourced from Gibco (Grand Island, NY, USA). All other chemicals and reagents were of the highest grade available and were obtained from local commercial sources.

Primary astrocytes culture

Astrocytes were isolated from the ventral mesencephalon (VM) of newborn Wistar rats as previously described by our lab ^{11,12}. In brief, the tissues were dissected from Wistar pups aged 1-2 days, and after the meninges were removed, the tissues were transferred to a tube containing cold D-Hank's solution. The pure tissue was mechanically dissociated and digested by incubation with 0.1% trypsin for 10 min at 37°C. The reaction was stopped by the addition of complete medium (DMEM/F12, 10% FBS with 100 U/mL penicillin and 100 U/mL streptomycin). The tissues were mechanically digested again by pipetting until full dissociation into single cells was accomplished. After centrifugation at 1000 rpm for 5 min, the pellets were resuspended in complete medium and plated in poly-D-lysine-coated 150 cm² flasks (5

brains/flask). After approximately 8-10 days, the confluent culture was placed horizontally on a shaker platform with medium covering the cells, shaken at 250 rpm for 18 h, and the medium that contained microglia was removed. The attached cells were trypsinized and re-plated at 33% of the original density. Cells were subsequently cultured until they reached 80% confluence. Purified astrocytes were more than 97% positive for GFAP and less than 1% positive for microglial surface molecule CD11b (data not shown). They were plated on poly-D-lysine coated 6-well or 24-well plates two days before the experiments.

Primary rat ventral mesencephalon (VM) neurons culture

Primary rat VM neurons culture were obtained from embryonic day 14-15 Wistar rats as described previously by our laboratory^{9, 40}. Briefly, VM tissues were dissected from embryonic day 14-15 rat brains and dissociated mechanically. Following centrifugation, cells were suspended in DMEM/F12 supplemented with 2% B27, 100 U/ml penicillin, and 100 µg/ml streptomycin and seeded on poly-D-lysine-coated coverslips or 12-well culture plates at a density of 6×10^5 cells/ml or 1.5×10^6 cells/ml, respectively. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ for 18 h, then, the medium was changed. Cultures were replenished with fresh medium 3 days later and used at day 6. Neuron purity was approximately 96% based on immunofluorescent staining with the specific neuronal marker MAP2. Approximately 2% of the neurons exhibited TH positivity, indicating DAergic neurons (data not shown).

Drug treatment

We dissolved 6-OHDA in a solution of 200 µg/ml L-ascorbic acid in 0.9% NaCl to create a stock concentration of 1 mM. We used a final concentration of 10 µM for experiments. To observe the effect of activation of HIF-1α and HIF-2α on cell metabolism, both the primary cultured astrocytes and VM neurons were divided into two groups: a control group and 6-OHDA group. In the subsequent inhibition experiment, primary cultured astrocytes were pretreated with BAY 87-2243 or HIF-2α inhibitor, and then 10 µM 6-OHDA. Astrocytes were divided into four groups: a control group, a 6-OHDA group, a HIF-1α or HIF-2α inhibitor group and a HIF-1α or HIF-2α inhibitor pretreatment group. In the control group, cells were treated with the basic medium, which had been used as the vehicle for the inhibitors and 6-OHDA. In the 6-OHDA group, cells were treated with 10 µM 6-OHDA for 24h. In the HIF-1α inhibitor pretreated group, cells were pretreated with 10 µM BAY 87-2243 for 48 h, and then co-treated with 10 µM BAY 87-2243 and 10 µM 6-OHDA for 24 h. In the HIF-2α inhibitor pretreatment group, cells were pretreated with 10 µM HIF-2α inhibitor for 24 h, and then co-treated with 10 µM HIF-2α inhibitor and 10 µM 6-OHDA for 24 h.

In the PKC experiments, primary cultured astrocytes were pretreated with 0.4 µM the PKC inhibitor BisI for 24 h, and then with either 0.2 µM of the PKC activator PMA or 10 µM 6-OHDA. To study the effect of PMA or 6-OHDA, astrocytes were divided into four groups: a control group, a BisI group, a BisI pretreatment group and a PMA or 6-OHDA group, respectively. In the control group, cells were treated with the basic medium, as described above.

In the ROS/RNS experiment, primary cultured astrocytes were pretreated with either 1mM the radical scavenger NAC or 1mM the inducible NO synthase (iNOS) inhibitor L-NAME for 30 min, followed by application of 10 μ M 6-OHDA. Astrocytes were divided into four groups: a control group, a 6-OHDA group, a NAC pretreatment group, and a L-NAME pretreatment group. In the control group, cells were treated with the basic medium.

Western blotting

Astrocytes and VM neurons were treated as described above and after three washes with cold phosphate-buffered saline, cells were lysed with lysis buffer containing 50 mM Tris HCl, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors (pepstatin 1 μ g/ml, aprotinin 1 μ g/ml, leupeptin 1 μ g/ml) for 30 min on ice. The insoluble material was removed by centrifugation (12000 rpm, 20 min, 4°C). Protein concentration was determined by the Bradford assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). A total of 30 μ g of protein was separated using 8% SDS-polyacrylamide gels and transferred to Polyvinylidene Fluoride (PVDF) membranes. After blocking with 10% non-fat milk at room temperature for 2 h, the membranes were incubated with rabbit anti-rat HIF-1 α (1:1000), or HIF-2 α (1:1000), or DMT1 (1:800), or FPN1 (1:800) or PKC δ (1:800) and *p*-PKC δ (1:800) antibodies over night at 4°C. Anti-rabbit secondary antibody conjugated to horseradish peroxidase was used at 1:10000 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were probed with anti- β -actin antibody (1:10000) as a loading control. Cross-reactivity was visualized using ECL Western blotting detection reagents and analyzed by scanning densitometry using a UVP BioDoc-Imaging System (UVP, Upland, CA, USA).

Calcein loading of cells and ferrous iron influx and efflux assay

The ferrous iron traffic of astrocytes was determined by measuring the quenching or reversing of calcein fluorescence, as previously described ^{11, 12}. Cells were seeded onto coverslips and grown in serum-free medium with 10 μ M 6-OHDA for 24 h, after HIF-1 α inhibitor pretreatment for 48 h and HIF-2 α pretreatment for 24 h. Then they were incubated with calcein-AM (0.5 μ M final concentrations of calcein-AM) in Hepes-buffered saline (HBS; 10 mM Hepes, 150 mM NaCl, pH 7.4) for 30 min at 37°C. For iron influx into astrocytes, cells were perfused with 1 mM ferrous iron (ferrous sulfate in ascorbic acid solution; 1:44 molar ratio, pH 6.0). Calcein fluorescence was recorded using an Olympus FV5 confocal microscope at 488 nm excitation and 525 nm emission wavelengths and fluorescence intensity was measured 10 times at 3 min intervals.

For the iron efflux assay, the cover slip was washed with HBS three times after 30 min incubation with 1 mM ferrous iron, and then exposed to 1 mM DFO, a membrane-impermeable, strong and specific iron chelator. The intracellular iron was drained out to the medium, leading to an increase in calcein fluorescence which was recorded as described above. The fluorescence intensity reported represents the

mean value of 35-40 separate cells from four fields monitored at ×20 magnification at each time point using the Fluoview 5.0 Software (Leica Microsystems Inc, Buffalo Grove, IL, USA) .

Statistical analysis

Results are presented as mean ± SEM. Student's two-tailed *t*-test was used to compare the difference of means in experiments with only two groups. A one-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was used to compare differences of means in experiments with more than two groups. Influx and efflux studies were carried out using a repeated measures ANOVA. A probability of $P < 0.05$ was taken to indicate statistical significance.

Results

6-OHDA treatment activates HIF-1α and HIF-2α in primary cultured astrocytes, but not in VM neurons

To investigate if HIFs are specifically activated in astrocytes treated with 6-OHDA, we used Western blots to quantify HIF-1α and HIF-2α expression in primary cultured astrocytes and VM neurons treated with 6-OHDA. Increased expressions of HIF-1α and HIF-2α was observed in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h compared to control (Figure. 1a, $P < 0.01$; Figure. 1b, $P < 0.05$). However, protein levels of HIF-1α and HIF-2α remained unchanged in VM neurons after application of 6-OHDA compared to control (Figure. 1c and Figure. 1d; $P > 0.05$). These results indicate that 6-OHDA treatment activates HIF-1α and HIF-2α in primary cultured astrocytes, but not in VM neurons.

6-OHDA-induced overexpressions of DMT1 and FPN1 in cultured astrocytes do not require HIF-1α upregulation

We next tested the effects of BAY 87-2243, a potent and selective HIF-1α inhibitor, on primary cultured astrocytes. Cells were pretreated with 10 μM BAY 87-2243 for 48 h, and then co-treated with 10 μM BAY 87-2243 and 10 μM 6-OHDA for 24 h. Expression of HIF-1α increased in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with the control group (Figure. 2a; $P < 0.05$). In contrast, the expression of HIF-1α decreased in primary cultured astrocytes treated with 10 μM BAY 87-2243, compared with the control group (Figure. 2a; $P < 0.05$). Furthermore, BAY 87-2243 pretreatment decreased the expression of HIF-1α in 6-OHDA-treated cells, compared with the 6-OHDA group (Figure. 2a; $P < 0.05$). To further investigate whether HIF-1α modulates the expressions of DMT1 and FPN1 induced by 6-OHDA in primary cultured astrocytes, measured the expressions of DMT1 and FPN1 after inhibiting HIF-1α with BAY 87-2243 (Figure. 2b and Figure. 2c). We observed that DMT1 and FPN1 expression increased in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with the control group (Figure. 2b, $P < 0.01$; Figure. 2c, $P < 0.05$). However, the expressions of DMT1 and FPN1 remained unchanged in astrocytes treated with 10 μM BAY 87-2243 alone, compared with the control group (Figure. 2b; Figure. 2c; $P > 0.05$). Expressions of DMT1 and FPN1 did not change in the BAY 87-2243 pretreatment group, compared to the 6-OHDA group (Figure. 2B; Figure. 2C; $P > 0.05$). Overall, these results demonstrate that BAY 87-2243 suppressed expression of HIF-1α and inhibited the overexpression of HIF-1α in primary

cultured astrocytes treated with 6-OHDA. However, BAY 87-2243 did not inhibit the up-regulation of DMT1 and FPN1 by 6-OHDA, suggesting that HIF-1 α does not contribute to these processes.

HIF-2 α upregulation is required for 6-OHDA induced overexpressions of DMT1 and FPN1

We next investigated the effects of a potent and selective inhibitor of HIF-2 α , which acts specifically on the subunits of HIF-2 α . Primary cultured astrocytes were pretreated with 10 μ M HIF-2 α inhibitor for 24 h, and then co-treated with 10 μ M HIF-2 α inhibitor and 10 μ M 6-OHDA for 24 h. Expression of HIF-2 α increased in primary cultured astrocytes treated with 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 3a; $P < 0.05$). In contrast, treatment with 10 μ M HIF-2 α inhibitor decreased the expression of HIF-2 α in primary cultured astrocytes, compared with the control group (Figure. 3a; $P < 0.05$). Furthermore, HIF-2 α inhibitor pretreatment decreased the expression of HIF-2 α in 6-OHDA-treated cells, compared with the 6-OHDA group (Figure. 3a; $P < 0.01$). To obtain direct evidence about whether HIF-2 α contributes to modulating the expression of DMT1 and FPN1 in activated primary cultured astrocytes, we measured expression levels of DMT1 and FPN1 in primary cultured astrocytes treated with HIF-2 α inhibitor. Expressions of DMT1 and FPN1 increased in primary cultured astrocytes treated with 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 3b, $P < 0.05$; Figure. 3c, $P < 0.01$). However, DMT1 and FPN1 expression decreased in primary cultured astrocytes treated with 10 μ M HIF-2 α inhibitor, compared to the control group (Figure. 3b, $P < 0.01$; Figure. 3c, $P < 0.05$). Similarly, HIF-2 α inhibitor pretreatment decreased the expressions of DMT1 and FPN1 in 6-OHDA-treated cells, compared with the 6-OHDA group (Figure. 3b, $P < 0.05$; Figure. 3c, $P < 0.01$). In summary, the HIF-2 α inhibitor suppressed expression of HIF-2 α , as expected, and prevented the overexpression of HIF-2 α in primary cultured astrocytes by 6-OHDA. These results suggest that HIF-2 α modulates the influence of 6-OHDA on DMT1 and FPN1 expression.

HIF-2 α was involved in the increased ferrous iron influx and efflux in 6-OHDA-treated primary cultured astrocytes

The fluorescent dye calcein was used to monitor ferrous iron influx into primary astrocytes during a 1 mM ferrous iron perfusion. The fluorescence intensity declined gradually inside cells, indicating a transmembrane ferrous iron influx (Figure. 4). We investigated the roles of astrocytes in regulating iron homeostasis under oxidative conditions. In agreement with our previous data for primary astrocytes¹¹, cells treated with 10 μ M 6-OHDA showed a more rapid fluorescence quenching and a decrease in fluorescence intensity compared with controls. However, pretreatment with 10 μ M HIF-2 α inhibitor for 24 h fully blocked this process and produced a fluorescence intensity similar to control, indicating that the HIF-2 α inhibitor suppresses the increased ferrous iron influx caused by 6-OHDA.

For the iron efflux assay, we used DFO, a membrane-impermeable, potent, specific iron chelator. When cells were perfused with 1 mM DFO, a marked increase in fluorescence intensity was observed in the 10 μ M 6-OHDA-treated group. These results indicate that 6-OHDA enhances iron transportation rate in primary cultured astrocytes. However, pretreatment with 10 μ M HIF-2 α inhibitor for 24 h fully blocked this

process and yielded a fluorescence intensity similar to control levels, indicating that the HIF-2 α inhibitor suppresses the ferrous iron efflux caused by 6-OHDA.

PKC pathway was involved in the 6-OHDA induced activation of HIF-2 α in primary cultured astrocytes.

To investigate if PKC pathway is involved in the activation of HIF-2 α in astrocytes, we quantify HIF-2 α expression in primary cultured astrocytes treated with PMA or 6-OHDA. Primary cultured astrocytes were pretreated with 0.4 μ M Bisl for 24 h, and then co-treated with 0.4 μ M Bisl and 0.2 μ M PMA or 10 μ M 6-OHDA for 24 h. Expression of HIF-2 α increased after treatment of primary astrocytes with 0.2 μ M PMA or 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 5a; Figure. 5b; $P < 0.05$). However, HIF-2 α expression did not change when astrocytes were treated with 0.4 μ M Bisl for 24 h, compared to the control group (Figure. 5a; Figure. 5b; $P > 0.05$). Next, primary cultured astrocytes were pretreated with 0.4 μ M Bisl for 24 h, and then co-treated with 0.4 μ M Bisl and 0.2 μ M PMA or 10 μ M 6-OHDA for 24 h. Pretreatment with Bisl decreased HIF-2 α expression in PMA-treated cells or 6-OHDA-treated cells, compared with the PMA or 6-OHDA group (Figure. 5a; Figure. 5b; $P < 0.05$), but expression of HIF-2 α did not differ from the control (Figure. 5a; Figure. 5b; $P > 0.05$).

We next examined whether expression levels of DMT1 and FPN1 increase following HIF-2 α activation due to HIF/HRE binding. To confirm that the activated HIF-2 α is functional, DMT1 and FPN1 expression were investigated. The expression of DMT1 increased in primary cultured astrocytes treated with 0.2 μ M PMA or 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 5c; Figure. 5e; $P < 0.05$). In contrast, the expression of DMT1 did not change in primary cultured astrocytes after 0.4 μ M Bisl treatment, compared with the control group (Figure. 5c; Figure. 5e; $P > 0.05$). However, Bisl pretreatment decreased the expression of DMT1 in PMA-treated cells or 6-OHDA-treated cells, compared with the PMA or 6-OHDA group (Figure. 5c; Figure. 5e; $P < 0.05$), but expression of DMT1 did not differ from control (Figure. 5c; Figure. 5e; $P > 0.05$).

The expression of FPN1 increased in primary cultured astrocytes treated with 0.2 μ M PMA or 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 5d; Figure. 5f; $P < 0.05$). However, the expression of FPN1 did not change in astrocytes treated with 0.4 μ M Bisl, compared to the control group (Figure. 5d; Figure. 5f; $P > 0.05$). In contrast, pretreatment with Bisl decreased the expression of FPN1 in PMA-treated cells or 6-OHDA-treated cells, compared with the PMA or 6-OHDA group (Figure. 5d; Figure. 5f; $P < 0.05$), but expression of FPN1 did not differ from control (Figure. 5d; Figure. 5f; $P > 0.05$). These results indicate that in primary cultured astrocytes, 6-OHDA upregulates HIF-2 α expression via PKC activation and that the activated HIF-2 α is functional.

ROS and NO were involved in the 6-OHDA induced activation of HIF-2 α in primary cultured astrocytes.

Since both oxidative stress and NO activate HIFs, we assumed that upregulation of HIF-2 α may be initiated by intracellular oxidative stress alone or in combination with NO production induced by pro-inflammatory cytokines. To test this hypothesis, we investigated the expression of HIF-2 α in 6-OHDA-treated primary cultured astrocytes after pretreatment with the radical scavenger NAC and the inducible

NO synthase (iNOS) inhibitor L-NAME. Expression of HIF-2 α significantly increased after primary cultured astrocytes were treated with 10 μ M 6-OHDA for 24 h, compared with the control group (Figure. 6a; $P < 0.01$). However, pretreatment with 1 mM NAC or 1 mM L-NAME decreased the expression of HIF-2 α in 6-OHDA-treated primary cultured astrocytes, compared with 6-OHDA group (Figure. 6a; $P < 0.05$). However, expression of HIF-2 α remained unchanged in both the NAC pretreatment group and the L-NAME pretreatment group compared with the control group (Figure. 6a; $P > 0.05$).

To confirm that the HIF-2 α expressed is functional, DMT1 and FPN1 expression were quantified. We found that the expressions of DMT1 and FPN1 increased in 10 μ M 6-OHDA treated primary cultured astrocytes, compared with the control group (Figure. 6b, $P < 0.05$; Figure. 6c, $P < 0.05$). However, pretreatment with either NAC (Figure. 6b, $P < 0.05$; Figure. 6c, $P < 0.05$) or L-NAME (Figure. 6b, $P < 0.01$; Figure. 6c, $P < 0.05$) decreased the expression of DMT1 and FPN1 in 6-OHDA-treated cells, compared with the 6-OHDA group. Expression of DMT1 and FPN1 remained unchanged when cells were pretreated with either NAC or L-NAME, compared with the control group (Figure. 6b, $P > 0.05$; Figure. 6c, $P > 0.05$). These results indicate that in primary cultured astrocytes, ROS and NO upregulate HIF-2 α expression and that the HIF-2 α expressed is functional.

ROS and NO were involved in the activation of PKC δ phosphorylation by 6-OHDA in primary cultured astrocytes.

To further study the mechanism about the activation of HIF-2 α by 6-OHDA in primary cultured astrocytes. Used western blots, we observed the level of PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes after pretreatment with NAC and L-NAME. PKC δ phosphorylation increased after primary cultured astrocytes were treated with 10 μ M 6-OHDA for 24 h, compared with the control group ($P < 0.05$). Pretreatment with NAC decreased PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes, compared with the 6-OHDA group ($P < 0.05$). Pretreatment with 1 mM L-NAME decreased PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes, compared with the 6-OHDA group ($P < 0.05$, Fig.7). These results indicated that ROS and NO were involved in the activation of PKC δ phosphorylation by 6-OHDA in primary cultured astrocytes.

Discussion

The results of the present study indicated that protein levels of HIF-1 α and HIF-2 α increased in astrocytes, but not in dopaminergic neurons, after treatment with 6-OHDA. We also found that HIF-2 α regulated the expressions of DMT1 and FPN1 which are responsible for regulating iron transport in astrocytes. Furthermore, these data suggests that the upregulation of HIF-2 α expression by 6-OHDA occurs via PKC activation. Finally, we propose that 6-OHDA induced ROS and RNS to activate HIF-2 α .

In the present study, we observed that the protein levels of HIF-1 α and HIF-2 α were increased after astrocytes were treated with 6-OHDA but not in dopaminergic neurons. This further supports our hypothesis that the mechanisms of iron metabolism in astrocytes is different from that in neurons, which

is regulated by IRP1⁷⁻¹⁰. Subsequently, we investigated whether iron metabolism in astrocytes is similar to that in the small intestinal epithelial cell, which is dependent on HIF-2 α but not HIF-1 α . We observed that 6-OHDA activated HIF-1 α and HIF-2 α , and significantly increased the expressions of DMT1 and FPN1 which lead to an increased iron uptake and efflux, respectively. To dissect the roles of each HIF subtype, we used the selective HIF-1 α inhibitor BAY 87-2243, which shows a dose-dependent *in vivo* antitumor efficacy in the H460 lung tumor xenograft model, accompanied by a suppression of HIF-1 α protein and HIF-1 target gene expression without any sign of toxicity or body weight loss. Further mechanism-of-action analyses have revealed that BAY 87-2243 exerts its effect on the HIF pathway by blocking mitochondrial complex I activity and thereby reducing HIF protein levels under hypoxia⁴¹. The HIF-2 α inhibitor used in this study is a cell-permeable thienylhydrazone compound that suppresses the translation of the cellular HIF-2 α messenger RNA. It exhibits no effect on the transcription of HIF-2 α mRNA or the stability of HIF-2 α mRNA and protein. Furthermore, it has been shown to reduce protein levels of HIF-2 α and HIF-2 α target genes in 786-O cells. Prior studies have reported that 10 μ M is the best concentration to inhibit the expression of only the 2 α isoform of HIF⁴².

We applied BAY 87-2243 and HIF-2 α inhibitor separately to specifically inhibit expression of HIF-1 α and HIF-2 α in astrocytes, respectively. We additionally quantified the effect of these inhibitors on expression of DMT1 and FPN1. Our findings indicate that HIF-2 α , but not HIF-1 α , mediates the regulation of DMT1 and FPN1 by 6-OHDA in astrocytes. However, it is unclear why preventing the 6-OHDA-induced activation of HIF-1 α with inhibitors did not have the effect of regulating iron metabolism. We hypothesize that HIF-1 α has other important functions in astrocytes and discuss the possible regulating function of HIF-1 α in astrocytes.

Previous studies have suggested that PKC modulates the HIF pathway in the human cervical cancer cell line HeLa⁴³. These studies highlight the roles of PKC isoforms as additional, discrete modulators of hypoxia-stimulated HIF accumulation through different signaling pathways in cancer cells^{44,45}. Studies indicate that hypoxia activates PKC δ , in addition, both in animal models and human bronchial epithelial BEAS-2B cell lines, PKC δ via PI3K/ AKT/ mTOR/ HIF pathway activated allergic inflammatory reaction⁴⁴. In PC12 and HEK293 cell lines, the activation of PKC δ increased the translation of HIF-1 α through mTOR pathway⁴⁵. Under hypoxia, PKC δ translocated into cell, leading to transcriptional activation of HIF-1 α in RIF cell lines⁴⁶. Kim et al.³⁵ indicated that among a large number of PKC subunits, PKC δ and PKC α regulated the aggregation of HIF-1 α in both HeLa cell lines, PC3 or HT1080 tumor cell lines⁴³. Zhang et al.⁴⁷ indicated that in liver cancer cells, the decreased PKC δ was accompanied by the down-regulation of HIF-2 α . On the other hand, they confirmed that HIF-2 α affected the phosphorylation level of PKC δ and promoted PKC δ migration by regulating CDCP1 protein. In our previous study, we demonstrated 6-OHDA-induced PKC δ and PKC ζ activation in dopaminergic MES23.5 cells⁴⁸. We speculated that 6-OHDA might activate HIF-2 α via PKC activation to affect iron metabolism in astrocytes. To study the mechanisms underlying the activation of HIF-2 α by 6-OHDA, we used the PKC activator PMA and the PKC inhibitor BisI and measured the expression of HIF-2 α . The results showed that PMA could activate HIF-2 α and BisI to inhibit the activation of HIF-2 α by PMA. The activation of DMT1 and FPN1 by PMA also could be

inhibited by BisI. Furthermore, we observed that BisI also inhibits the activation of HIF-2 α by 6-OHDA and prevents upregulation of DMT1 and FPN1 by 6-OHDA. These results provide evidence that 6-OHDA activates HIF-2 α via PKC activation, with HIF-2 α regulating the expression of DMT1 and FPN1.

Increased ROS/RNS levels induce stabilization, accumulation and activation of HIFs even in normoxia in cancer cells. Studies have shown that ROS/RNS regulate HIF-1 α activity through the PI3K and MAPK cascade pathways in different tumor types^{49, 50}. Yang YC et al.⁵¹ indicated that apoptosis induced by ROS through activation of PKC δ and mitochondrial translocation. When cells were treated with H₂O₂, PKC δ was phosphorylated. PKC δ were involved in ROS mediated mitochondrial dysfunction. L. Fão & I. Mota⁵² indicated that HT22 cells were treated with H₂O₂, PKC δ was phosphorylated at Tyr311. Therefore, we suggest that 6-OHDA induces ROS and/or RNS to activate HIF-2 α , which may be associated with ROS and NO activation of PKC δ , affects iron metabolism in astrocytes. In the present study, we used the radical scavenger NAC and the inducible iNOS inhibitor L-NAME while measuring the expression of HIF-2 α in 6-OHDA-treated astrocytes. We found that NAC and L-NAME both block the activation of HIF-2 α by 6-OHDA and that the activation of DMT1 and FPN1 by 6-OHDA are also inhibited. NAC and L-NAME both block the activation of PKC δ phosphorylation by 6-OHDA in primary cultured astrocytes. These results demonstrate that 6-OHDA-induced ROS and RNS activate HIF-2 α to influence iron metabolism in astrocytes, and ROS and NO are involved in the activation of PKC δ phosphorylation by 6-OHDA.

In summary, our present study demonstrates that HIFs play a key role in iron metabolism in astrocytes. We found that HIF-2 α , but not HIF-1 α , regulates the activation of DMT1 and FPN1 by 6-OHDA to alter the rate of iron transport. Furthermore, our data indicate that PKC pathway is involved in 6-OHDA activation of HIF-2 α in astrocytes, which may be associated with ROS and NO activation of PKC δ , thus altering iron metabolism in astrocytes (Figure. 8).

Conclusion

In conclusion, astrocytes are functionally similar to peripheral small intestinal epithelial cells, which in addition to their own use of iron, supply this micronutrient to neurons, oligodendrocytes and microglia through DMT1-mediated iron uptake and FPN1-mediated iron release. They play a vital role in transporting iron across the BBB and maintaining iron homeostasis in the brain. Dysfunctional iron uptake and transport in astrocytes disrupts iron homeostasis in the brain, leading to neurodegenerative diseases such as PD. However, our current understanding of iron transport and metabolism in the brain is much poorer than our understanding of these processes in the liver, spleen and intestine. It is therefore important to conduct further studies to better understand the important roles that astrocytes play in maintaining brain iron homeostasis and protecting the brain from oxidative stress injury induced by iron. In the future, astrocytes may become novel targets to prevent and control iron-related neurodegenerative diseases.

List Of Abbreviations

PD, Parkinson's disease; SN, substantia nigra; 6-OHDA, 6-hydroxydopamine; DMT1, divalent metal transporter 1; FPN1, ferroportin1; HIFs, Hypoxia-inducible factors; HIF-2 α , Hypoxia-inducible factor-2 α ; HREs, hypoxic response elements; ARNT, arylhydrocarbon receptor nuclear translocator; IRP1, iron regulatory proteins 1; IRE, iron responsive element; TfR-2, transferrin receptor-2; BBB, blood-brain barrier; CNS, central nervous system; PKC, protein kinase; ROS, Reactive oxygen species; RNS, reactive nitrogen species; H₂O₂, hydrogen peroxide; NO, nitric oxide; PI3K, phosphatidylinositol 3-kinase; MAPK, Mitogen Activated Protein Kinase; Bisl, bisindolylmaleimide I hydrochloride; PMA, phorbol 12-myristate-13-acetate; NAC, N-acetyl-L-cysteine; L-NAME, N ω -Nitro-L-arginine methyl ester hydrochloride; FeSO₄.7H₂O, ferrous Sulfate sulfate heptahydrate; DFO, deferoxamine; DMSO, dimethyl sulfoxide; DMEM/F12, Dubecco's modified Eagle's medium; FBS, fetal bovine serum; VM, ventral mesencephalon; PMSF, phenylmethanesulfonyl fluoride; PVDF, Polyvinylidene Fluoride; ANOVA, A one-way analysis of variance.

Declarations

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Availability of data and material

All data analyzed in this study are included within the article.

Author's Contributions

Jun Wang and Junxia Xie designed the study, did interpretation of data, and revised the manuscript. Manman Xu wrote the manuscript. Manman Xu and Zhiqiang Chen performed experiments and analyzed the data. Xizhen Ma and Lingyu Xu assisted with experimental techniques, analyzed the data. All authors read and approved the final manuscript.

Ethics approval

Animals were housed and tested in compliance with the institutional guidelines at Medical College of Qingdao University.

Consent for publication

The authors declare that they have no conflicts of interest to report.

Competing interests

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Authors' information

Information for all the co-authors is listed in the title page.

References

1. Zecca L, Stroppolo A, Gatti A, et al. The role of iron and copper molecules in the neuronal vulnerability of locus coeruleus and substantia nigra during aging. *Proceedings of the National Academy of Sciences of the United States of America* 2004;101(26):9843-9848.
2. Berg D, Hochstrasser H. Iron metabolism in Parkinsonian syndromes. *Movement disorders : official journal of the Movement Disorder Society* 2006;21(9):1299-1310.
3. Teismann P, Schulz JB. Cellular pathology of Parkinson's disease: astrocytes, microglia and inflammation. *Cell and tissue research* 2004;318(1):149-161.
4. Oshiro S, Kawamura K, Zhang C, et al. Microglia and astroglia prevent oxidative stress-induced neuronal cell death: implications for aceruloplasminemia. *Biochimica et biophysica acta* 2008;1782(2):109-117.
5. Hare DJ, Double KL. Iron and dopamine: a toxic couple. *Brain : a journal of neurology* 2016;139(Pt 4):1026-1035.
6. Xu H, Wang Y, Song N, Wang J, Jiang H, Xie J. New Progress on the Role of Glia in Iron Metabolism and Iron-Induced Degeneration of Dopamine Neurons in Parkinson's Disease. *Frontiers in molecular neuroscience* 2017;10:455.
7. Wang J, Jiang H, Xie JX. Ferroportin1 and hephaestin are involved in the nigral iron accumulation of 6-OHDA-lesioned rats. *The European journal of neuroscience* 2007;25(9):2766-2772.
8. Jiang H, Song N, Xu H, Zhang S, Wang J, Xie J. Up-regulation of divalent metal transporter 1 in 6-hydroxydopamine intoxication is IRE/IRP dependent. *Cell research* 2010;20(3):345-356.
9. Song N, Wang J, Jiang H, Xie J. Ferroportin 1 but not hephaestin contributes to iron accumulation in a cell model of Parkinson's disease. *Free radical biology & medicine* 2010;48(2):332-341.
10. Junxia X, Hong J, Wenfang C, Ming Q. Dopamine release rather than content in the caudate putamen is associated with behavioral changes in the iron rat model of Parkinson's disease. *Experimental neurology* 2003;182(2):483-489.
11. Zhang HY, Wang ND, Song N, et al. 6-Hydroxydopamine promotes iron traffic in primary cultured astrocytes. *Biometals : an international journal on the role of metal ions in biology, biochemistry, and medicine* 2013;26(5):705-714.

12. Zhang HY, Song N, Jiang H, Bi MX, Xie JX. Brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor inhibit ferrous iron influx via divalent metal transporter 1 and iron regulatory protein 1 regulation in ventral mesencephalic neurons. *Biochimica et biophysica acta* 2014;1843(12):2967-2975.
13. Zhang W, Dourado DF, Mannervik B. Evolution of the active site of human glutathione transferase A2-2 for enhanced activity with dietary isothiocyanates. *Biochimica et biophysica acta* 2015;1850(4):742-749.
14. Zenaro E, Piacentino G, Constantin G. The blood-brain barrier in Alzheimer's disease. *Neurobiology of disease* 2017;107:41-56.
15. Dringen R, Bishop GM, Koeppe M, Dang TN, Robinson SR. The pivotal role of astrocytes in the metabolism of iron in the brain. *Neurochemical research* 2007;32(11):1884-1890.
16. Song N, Wang J, Jiang H, Xie J. Astroglial and microglial contributions to iron metabolism disturbance in Parkinson's disease. *Biochim Biophys Acta Mol Basis Dis* 2018;1864(3):967-973.
17. Benarroch EE. Brain iron homeostasis and neurodegenerative disease. *Neurology* 2009;72(16):1436-1440.
18. Ward RJ, Zucca FA, Duyn JH, Crichton RR, Zecca L. The role of iron in brain ageing and neurodegenerative disorders. *Lancet Neurol* 2014;13(10):1045-1060.
19. Healy S, McMahon JM, FitzGerald U. Modelling iron mismanagement in neurodegenerative disease in vitro: paradigms, pitfalls, possibilities & practical considerations. *Prog Neurobiol* 2017;158:1-14.
20. Mirza B, Hadberg H, Thomsen P, Moos T. The absence of reactive astrogliosis is indicative of a unique inflammatory process in Parkinson's disease. *Neuroscience* 2000;95(2):425-432.
21. Hagemeyer J, Geurts JJG, Zivadinov R. Brain iron accumulation in aging and neurodegenerative disorders. *Expert Rev Neurother* 2012;12(12):1467-1480.
22. Bartzokis G, Tishler TA, Lu PH, et al. Brain ferritin iron may influence age- and gender-related risks of neurodegeneration. *Neurobiol Aging* 2007;28(3):414-423.
23. Taylor M, Qu A, Anderson ER, et al. Hypoxia-inducible factor-2alpha mediates the adaptive increase of intestinal ferroportin during iron deficiency in mice. *Gastroenterology* 2011;140(7):2044-2055.
24. Flamme I, Frohlich T, von Reutern M, Kappel A, Damert A, Risau W. HRF, a putative basic helix-loop-helix-PAS-domain transcription factor is closely related to hypoxia-inducible factor-1 alpha and developmentally expressed in blood vessels. *Mechanisms of development* 1997;63(1):51-60.
25. Tian H, McKnight SL, Russell DW. Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes & development* 1997;11(1):72-82.
26. Gu YZ, Moran SM, Hogenesch JB, Wartman L, Bradfield CA. Molecular characterization and chromosomal localization of a third alpha-class hypoxia inducible factor subunit, HIF3alpha. *Gene expression* 1998;7(3):205-213.
27. Wiener CM, Booth G, Semenza GL. In vivo expression of mRNAs encoding hypoxia-inducible factor 1. *Biochemical and biophysical research communications* 1996;225(2):485-488.

28. Wiesener MS, Jurgensen JS, Rosenberger C, et al. Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2003;17(2):271-273.
29. Duan C. Hypoxia-inducible factor 3 biology: complexities and emerging themes. *American journal of physiology Cell physiology* 2016;310(4):C260-269.
30. Meyron-Holtz EG, Ghosh MC, Rouault TA. Mammalian tissue oxygen levels modulate iron-regulatory protein activities in vivo. *Science (New York, NY)* 2004;306(5704):2087-2090.
31. Mastrogiannaki M, Matak P, Keith B, Simon MC, Vulont S, Peyssonnaud C. HIF-2alpha, but not HIF-1alpha, promotes iron absorption in mice. *The Journal of clinical investigation* 2009;119(5):1159-1166.
32. Wagner BP, Nedelcu J, Martin E. Delayed postischemic hypothermia improves long-term behavioral outcome after cerebral hypoxia-ischemia in neonatal rats. *Pediatric research* 2002;51(3):354-360.
33. Vannucci RC, Towfighi J, Vannucci SJ. Hypoxic preconditioning and hypoxic-ischemic brain damage in the immature rat: pathologic and metabolic correlates. *Journal of neurochemistry* 1998;71(3):1215-1220.
34. Datta K, Li J, Bhattacharya R, Gasparian L, Wang E, Mukhopadhyay D. Protein kinase C zeta transactivates hypoxia-inducible factor alpha by promoting its association with p300 in renal cancer. *Cancer Res* 2004;64(2):456-462.
35. Kim H, Na Y-R, Kim SY, Yang EG. Protein Kinase C Isoforms Differentially Regulate Hypoxia-Inducible Factor-1 α Accumulation in Cancer Cells. *J Cell Biochem* 2016;117(3):647-658.
36. Giorgi C, Agnoletto C, Baldini C, et al. Redox control of protein kinase C: cell- and disease-specific aspects. *Antioxid Redox Signal* 2010;13(7):1051-1085.
37. Galanis A, Pappa A, Giannakakis A, Lanitis E, Dangaj D, Sandaltzopoulos R. Reactive oxygen species and HIF-1 signalling in cancer. *Cancer Lett* 2008;266(1):12-20.
38. Movafagh S, Crook S, Vo K. Regulation of hypoxia-inducible factor-1a by reactive oxygen species: new developments in an old debate. *J Cell Biochem* 2015;116(5):696-703.
39. Wang W, Song N, Zhang H, Xie J, Wang J. 6-Hydroxydopamine upregulates iron regulatory protein 1 by activating certain protein kinase C isoforms in the dopaminergic MES23.5 cell line. *Int J Biochem Cell Biol* 2012;44(11):1987-1992.
40. Wang J, Song N, Jiang H, Wang J, Xie J. Pro-inflammatory cytokines modulate iron regulatory protein 1 expression and iron transportation through reactive oxygen/nitrogen species production in ventral mesencephalic neurons. *Biochimica et biophysica acta* 2013;1832(5):618-625.
41. Ellinghaus P, Heisler I, Unterschemmann K, et al. BAY 87-2243, a highly potent and selective inhibitor of hypoxia-induced gene activation has antitumor activities by inhibition of mitochondrial complex I. *Cancer medicine* 2013;2(5):611-624.
42. Zimmer M, Ebert BL, Neil C, et al. Small-molecule inhibitors of HIF-2a translation link its 5'UTR iron-responsive element to oxygen sensing. *Molecular cell* 2008;32(6):838-848.

43. Kim H, Na YR, Kim SY, Yang EG. Protein Kinase C Isoforms Differentially Regulate Hypoxia-Inducible Factor-1alpha Accumulation in Cancer Cells. *Journal of cellular biochemistry* 2016;117(3):647-658.
44. Choi YH, Jin GY, Li LC, Yan GH. Inhibition of protein kinase C delta attenuates allergic airway inflammation through suppression of PI3K/Akt/mTOR/HIF-1 alpha/VEGF pathway. *PloS one* 2013;8(11):e81773.
45. Baek SH, Lee UY, Park EM, Han MY, Lee YS, Park YM. Role of protein kinase Cdelta in transmitting hypoxia signal to HSF and HIF-1. *Journal of cellular physiology* 2001;188(2):223-235.
46. Hui AS, Bauer AL, Striet JB, Schnell PO, Czyzyk-Krzeska MF. Calcium signaling stimulates translation of HIF-alpha during hypoxia. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 2006;20(3):466-475.
47. Cao M, Gao J, Zhou H, et al. HIF-2alpha regulates CDCP1 to promote PKCdelta-mediated migration in hepatocellular carcinoma. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine* 2016;37(2):1651-1662.
48. Wang W, Song N, Zhang H, Xie J, Wang J. 6-Hydroxydopamine upregulates iron regulatory protein 1 by activating certain protein kinase C isoforms in the dopaminergic MES23.5 cell line. *The international journal of biochemistry & cell biology* 2012;44(11):1987-1992.
49. Galanis A, Pappa A, Giannakakis A, Lanitis E, Dangaj D, Sandaltzopoulos R. Reactive oxygen species and HIF-1 signalling in cancer. *Cancer letters* 2008;266(1):12-20.
50. Gao X, Schottker B. Reduction-oxidation pathways involved in cancer development: a systematic review of literature reviews. *Oncotarget* 2017;8(31):51888-51906.
51. Yang YC, Tsai CY, Chen CL, et al. Pkcdelta Activation is Involved in ROS-Mediated Mitochondrial Dysfunction and Apoptosis in Cardiomyocytes Exposed to Advanced Glycation End Products (Ages). *Aging and disease* 2018;9(4):647-663.
52. Fao L, Mota SI, Rego AC. c-Src regulates Nrf2 activity through PKCdelta after oxidant stimulus. *Biochimica et biophysica acta Molecular cell research* 2019;1866(4):686-698.

Figures

Figure 1

The protein levels of HIF-1α and HIF-2α in the 6-OHDA-treated primary cultured astrocytes and VM neurons. A. Increased expression of HIF-1α was observed in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with control (**P < 0.01). B. Increased expression of HIF-2α was observed in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with control (*P < 0.05). C. The expression of HIF-1α did not change in primary cultured VM neurons treated with 10 μM 6-OHDA for

24 h, compared with control ($P > 0.05$). D. The expression of HIF-2 α did not change in primary cultured VM neurons treated with 10 μ M 6-OHDA for 24 h, compared with control ($P > 0.05$). $n = 6$ per group.

Figure 2

The protein levels of HIF-1 α , DMT1 and FPN1 in 6-OHDA-treated primary cultured astrocytes. A. The expression of HIF-1 α increased in primary cultured astrocytes treated with 10 μ M 6-OHDA for 24 h, compared with control (* $P < 0.05$). The expression of HIF-1 α decreased in primary cultured astrocytes treated with 10 μ M BAY 87-2243, compared with control (* $P < 0.05$). Pretreatment with BAY 87-2243 decreased the expression of HIF-1 α in 6-OHDA-treated cells, compared with the 6-OHDA group (# $P < 0.05$). B. The expression of DMT1 increased in primary cultured astrocytes treated with 10 μ M 6-OHDA for 24 h, compared with control (** $P < 0.01$). The expression of DMT1 remained unchanged in primary cultured astrocytes treated with 10 μ M BAY 87-2243, compared with the control ($P > 0.05$). The expression of DMT1 increased in the BAY 87-2243 pretreatment group, compared with the control (* $P < 0.05$). C. The expression of FPN1 increased in primary cultured astrocytes treated with 10 μ M 6-OHDA for 24 h, compared with the control (* $P < 0.05$). The expression of FPN1 remained unchanged in primary cultured astrocytes treated with 10 μ M BAY 87-2243, compared with the control ($P > 0.05$). The expression of FPN1 increased in the BAY 87-2243 pretreatment group, compared with the control (* $P < 0.05$). $n = 6$ per group.

Figure 3

The protein levels of HIF-2α, DMT1 and FPN1 in 6-OHDA-treated primary cultured astrocytes. A. The expression of HIF-2α increased in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with control (* $P < 0.05$). The expression of HIF-2α decreased in primary cultured astrocytes treated with 10 μM HIF-2α inhibitor, compared with control (* $P < 0.05$). Pretreatment with HIF-2α inhibitor decreased the expression of HIF-2α in 6-OHDA-treated cells, compared with the 6-OHDA group (## $P < 0.01$). B. The expression of DMT1 increased in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with control (* $P < 0.05$). The expression of HIF-2α decreased in primary cultured astrocytes treated with 10 μM HIF-2α inhibitor, compared with control (** $P < 0.01$). Pretreatment with HIF-2α inhibitor decreased the expression of HIF-2α in 6-OHDA-treated cells, compared with the 6-OHDA group (# $P < 0.05$). C. The expression of FPN1 increased in primary cultured astrocytes treated with 10 μM 6-OHDA for 24 h, compared with control (** $P < 0.01$). The expression of HIF-2α decreased in primary cultured astrocytes treated with 10 μM HIF-2α inhibitor, compared with control (* $P < 0.05$). Pretreatment with HIF-2α inhibitor decreased the expression of HIF-2α in 6-OHDA-treated cells, compared with the 6-OHDA group (## $P < 0.01$). $n = 6$ per group.

Figure 4

Ferrous iron influx and efflux in primary cultured astrocytes A. Fluorescence intensity was lower in the 6-OHDA group compared with the control group, indicating greater iron influx (a repeated measures ANOVA, $P < 0.05$). Fluorescence intensity was higher in the HIF-2 α +6-OHDA group compared with the 6-OHDA group, indicating less iron influx (a repeated measures ANOVA, $P < 0.01$). The HIF-2 α group compared with the control group (a repeated measures ANOVA, $P > 0.05$). The HIF-2 α +6-OHDA group compared with

control group (a repeated measures ANOVA, $P > 0.05$). B. Fluorescence intensity was higher in the 6-OHDA group compared with the control group, indicating greater iron efflux (a repeated measures ANOVA, $P < 0.01$). Fluorescence intensity was lower in the HIF-2 α +6-OHDA group compared with the 6-OHDA group, indicating less iron efflux (a repeated measures ANOVA, $P < 0.01$). The HIF-2 α group compared with the control group (a repeated measures ANOVA, $P > 0.05$); The HIF-2 α +6-OHDA group compared with the control group (a repeated measures ANOVA, $P > 0.05$). The fluorescence intensity represents the mean value of 35–40 separate cells monitored at $\times 20$ magnification at each time point. Data are presented as the mean $\pm$ SEM of six independent experiments.

Figure 5

The influence of PKC pathway on the expressions of HIF-2 α , DMT1 and FPN1 in primary cultured astrocytes. A. Expression of HIF-2 α increased after primary cultured astrocytes were treated with 0.2 μ M PMA for 24 h, compared with the control group (* $P < 0.05$). Pretreatment with PKC inhibitor BisI decreased

the expression of HIF-2 α in PMA-treated cells, compared with the PMA group ($\#P < 0.05$), but expression of HIF-2 α did not differ from control ($P > 0.05$). Expression of HIF-2 α remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with the control group ($P > 0.05$). B. Expression of HIF-2 α increased after primary cultured astrocytes were treated with 10 μ M 6-OHDA for 24 h, compared with the control group ($*P < 0.05$). Expression of HIF-2 α remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with the control ($P > 0.05$). Pretreatment with BisI (BisI+6-OHDA group) decreased the expression of HIF-2 α in 6-OHDA-treated cells, compared with the 6-OHDA group ($\#P < 0.05$), but expression of HIF-2 α did not differ from control ($P > 0.05$). C. The expression of DMT1 increased in 0.2 μ M PMA-treated primary cultured astrocytes, compared with control ($*P < 0.05$). Expression of DMT1 remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with the control ($P > 0.05$). Pretreatment with BisI decreased the expression of DMT1 in PMA-treated cells, compared with PMA alone ($\#P < 0.05$), but expression of DMT1 did not differ from control ($P > 0.05$). D. Expression of FPN1 increased in 0.2 μ M PMA-treated primary cultured astrocytes, compared with control ($*P < 0.05$). Expression of FPN1 remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with control ($P > 0.05$). Pretreatment with BisI decreased the expression of FPN1 in PMA-treated cells, compared with PMA alone ($\#P < 0.05$), but expression of FPN1 did not differ from control ($P > 0.05$). E. Expression of DMT1 increased in 10 μ M 6-OHDA-treated primary cultured astrocytes, compared with control ($*P < 0.05$). Expression of DMT1 remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with control ($P > 0.05$). Pretreatment with BisI decreased the expression of DMT1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#P < 0.05$), but expression of DMT1 did not differ from control ($P > 0.05$). F. Expression of FPN1 increased in 10 μ M 6-OHDA-treated primary cultured astrocytes, compared with control ($*P < 0.05$). Expression of FPN1 remained unchanged in primary cultured astrocytes treated with 0.4 μ M BisI, compared with control ($P > 0.05$). Pretreatment with BisI decreased the expression of FPN1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#P < 0.05$), but expression of FPN1 did not differ from control ($P > 0.05$). $n = 6$ per group.

Figure 6

ROS and NO were involved in the activation of HIF-2 α in primary cultured astrocytes. A. Expression of HIF-2 α increased after primary cultured astrocytes were treated with 10 μ M 6-OHDA for 24 h, compared with

control (** $P < 0.01$). Pretreatment with NAC decreased expression of HIF-2 α in 6-OHDA-treated primary cultured astrocytes, compared with 6-OHDA alone ($\#P < 0.05$), but expression of HIF-2 α did not differ from control ($P > 0.05$). Pretreatment with 1 mM L-NAME decreased expression of HIF-2 α in 6-OHDA-treated primary cultured astrocytes, compared with 6-OHDA alone ($\#P < 0.05$), but expression of HIF-2 α did not differ from control ($P > 0.05$). B. Expression of DMT1 increased in 10 μ M 6-OHDA-treated primary cultured astrocytes, compared with control (* $P < 0.05$). Pretreatment with NAC decreased expression of DMT1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#P < 0.05$), but expression of DMT1 did not differ from control ($P > 0.05$). Pretreatment with L-NAME decreased expression of DMT1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#\#P < 0.01$), but expression of DMT1 did not differ from control ($P > 0.05$). C. Expression of FPN1 increased in 10 μ M 6-OHDA-treated primary cultured astrocytes, compared with control (* $P < 0.05$). Pretreatment with NAC decreased expression of FPN1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#P < 0.05$), but expression of FPN1 did not differ from control ($P > 0.05$). Pretreatment with L-NAME decreased expression of FPN1 in 6-OHDA-treated cells, compared with 6-OHDA alone ($\#P < 0.05$), but expression of FPN1 did not differ from control ($P > 0.05$). $n = 6$ per group.

Figure 7

ROS and NO were involved in the activation of PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes. PKC δ phosphorylation increased after primary cultured astrocytes were treated with 10 μM 6-OHDA for 24 h, compared with the control group (* $P < 0.05$). Pretreatment with NAC decreased PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes, compared with the 6-OHDA group (# $P < 0.05$). Pretreatment with 1 mM L-NAME decreased PKC δ phosphorylation in 6-OHDA-treated primary cultured astrocytes, compared with the 6-OHDA group (# $P < 0.05$). $n=6$ per group.

Figure 8

Graphical summary about the effects of 6-OHDA on iron metabolism in astrocytes are mediated by HIF-2 α . HIF-2 α , regulates the activation of DMT1 and FPN1 by 6-OHDA to alter the rate of iron transport. PKC pathway is involved in 6-OHDA activation of HIF-2 α in astrocytes, which may be associated with ROS and NO activation of PKC δ . 6-OHDA: 6-hydroxydopamine; DMT1: divalent metal transporter 1; FPN1: ferroportin1; Dcytb: duodenal cytochrome b; Cp: ceruloplasmin; HIF-2 α : Hypoxia-inducible factor-2 α ; PKC: protein kinase; ROS: Reactive oxygen species; NO: nitric oxide; Fe^{2+} : ferrous; Fe^{3+} : ferric.