HIF-1 α -Mediated Neuroprotection on MPP+-Induced Apoptosis in SH-SY5Y Cells

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ABSTRACT

Objective: To demonstrate protective role of hypoxia-inducible-factor-1 α (HIF-1 α) in cells with mitochondrial dysfunction.

Background: Emerging evidence has suggested that HIF-1 α plays an important role in embryonic development of many mammalian organs including brain. Studies showed that midbrain neural precursor cells are impaired in mice lacking HIF-1 α , and vascular endothelial growth factor (*VEGF*) and erythropoietin (*EPO*), two target genes of HIF-1 α , exert neuroprotection in animal models of Parkinson's disease (PD). Mitochondrial dysfunction is one of the proposed pathogenic mechanisms of PD. Irons (such as Fe^{2+}), or other common environmental toxins (such as mitochondrial complex I inhibitor 1-methyl-4-phenylpyridinium ion (MPP^+), are believed to be key contributors to mitochondrial dysfunction. Since HIF-1 α is rapidly degraded *via* ubiquitin-proteasome system in a Fe^{2+} and/or oxygen (O_2)-dependent manner, low levels of HIF-1 α in PD are considered to be related to excessive Fe^{2+} and mitochondrial complex I inhibition-caused redistribution of O_2 to enzymes, such as prolyl hydroxylase (PHD), leading to an O_2-dependent degradation of HIF-1 α .

Design/Method: We transfected human dopaminergic SH-SY5Y cells with mutant HIF-1 α construct, in which the transcriptional activity of HIF-1 α is largely retained, or treated cells with compounds, such as (-)-epigallocatechin-3-gallate (EGCG) (25 μM) with iron chelating properties, to enhance HIF-1 α . To suppress HIF-1 α , cells were transfected with small interference RNA (siRNA) of HIF-1 α . The cells were then exposed to MPP^+ (5 mM) followed by cell viability measurement using live/dead assay kit and apoptosis analysis using immunoblotting and ELISA assay.

Results: We found that increased HIF-1 α by gene transfer or EGCG treatment protected against MPP^+-induced reduction of cell viability and increase of apoptosis in SH-SY5Y cells. In contrast, suppression of HIF-1 α predisposed cells to MPP^+-induced injury and blocked neuroprotection by EGCG.

Conclusion: We conclude that activation of HIF-1 α might be a novel therapeutic strategy for PD. The mechanisms involved in the neuroprotection of HIF-1 α and its neuroprotective roles *in vivo* will be further explored.

BACKGROUND

Hypoxia-inducible factor-1 α (HIF-1 α) is a transcription factor that activates the transcription of genes and is responsible for progression cell survival and proliferation. The level of HIF-1 α expression is determined by the rates of protein synthesis and the protein degradation. The synthesis of HIF-1 α can be stimulated by growth factors, cytokines and other signaling molecules *via* activation of the phosphatidylinositol 3-kinase (PI3K) or mitogen-activated protein kinase (MAPK) pathway *via* oxygen (O_2)-independent mechanisms; whereas, the degradation of HIF-1 α is regulated *via* Fe^{2+} and/or O_2-dependent enzyme prolyl hydroxylase (PHD) (1,2). The increase of Fe^{2+} and/or O_2 activates PHD, which may catalyse the hydroxylation of 2 key proline residues (P402 and P564) at the oxygen-dependent degradation (ODD) domain (the VHL recognition domain) of HIF-1 α , leading to rapid degradation of HIF-1 α *via* ubiquitin-proteasome system (UPS) (Figure 1).

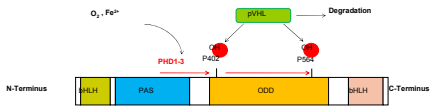


Figure 1. Degradation of HIF-1 α under normoxic condition (20% O2)

Aberrant iron accumulation, mitochondrial dysfunction and impairment of protein degradation system have been implicated in the pathogenesis of Parkinson's disease (PD). Although mitochondrial respiratory complex I inhibition can produce a state analogous to a prolonged hypoxia due to the reduction in the consumption of O_2, the impaired oxygen utilization will create the paradox of increased cellular O_2 availability for PHD and lead to a situation in which cells may fail to register hypoxia (3-6), causing the decrease of HIF-1 α (Figure 2). HIF-1α induces certain genetic expressions to participate in the process of cancer development and progression cell survival and proliferation, glucose metabolism, and drug resistance (7-9). Emerging evidence has suggested that HIF-1α is also an important gene for embryonic development of many mammalian organs including brain (10) and for midbrain neural precursor cells (11).

Considering the facts of neuronal death in PD and survival role of HIF-1 α , and the facts of metabolic characteristics of HIF-1α under the pathogenesis of PD that mitochondrial dysfunction and/or excessive Fe^{2+} may cause decrease of HIF-1 α , we speculated that enhancement of HIF-1α or compounds that may stabilize HIF-1α may alleviate neuronal death caused by excessive iron or mitochondrial injury under normoxic condition in PD

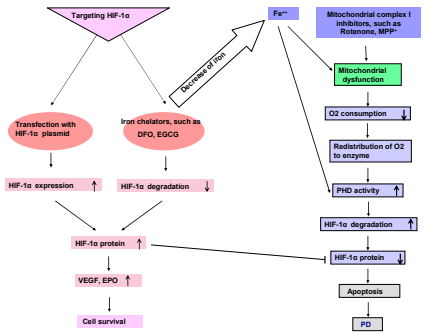


Figure 2. The metabolism and the roles of HIF-1α under the pathogenesis of PD. Mitochondrial complex I inhibitors, such as rotenone and MPP^+, and excessive irons, such as Fe^{2+}, may not only cause mitochondrial dysfunction, leading to apoptosis of cells related to PD, but also increase the activity of enzyme PHD, leading to the degradation of HIF-1α and then the reduction of HIF-1α protein level. HIF-1α gene transfection, iron chelators, such as DFO, EGCG, may increase HIF-1α protein level, through which its survival target genes, such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO), are induced, leading to cell survival. Additionally, iron chelators can chelate excessive Fe^{2+}, reduce the oxidative stress-induced mitochondrial injury,and stabilize HIF-1α due to the decreased degradation of HIF-1α.

METHODS

Cell culture and transfection SH-SY5Y cells were routinely grown in DMEM supplemented with 10% FBS and cultured at 37° C under humidified 5% CO_2 atmosphere. To enhance the expression of HIF-1 α , the cells were transfected with mutant HIF-1α construction, termed as "HIF-1 α / Δ ODD", in which the ODD domain has been deleted and the degradation of HIF-1 α will be blocked (kind gift from Dr. Zhen Fan, MD Anderson Cancer Center, Houston, TX) using lipofectamine 2000. To suppress the expression of HIF-1 α , cells were transfected with HIF-1α siRNA (RefSeq Number: NM_001530.3; NM_181054.2; siRNA ID: s6539; Ambio INC, Austin, TX, USA) using lipofectamine 2000. The pcDNA3.1 vector or #3 scrambled siRNA were used as negative control. To observe whether the neuroprotective role of iron chelator, such as (-)-epigallocatechin-3-gallate (EGCG), is mediated by HIF-1 α , cells were pretreated with EGCG (25 μM) followed by addition of rotenone or MPP^+ for another 24 h. To determine the effects of hypoxia on the induction of HIF-1α, the cells were cultured in hypoxia (1% O_2) conditions for 24 hr.

Immunoblotting assay After specific treatment, total proteins were isolated from the cells with mammalian tissue lysis/extraction reagent (Sigma-aldrich, St. Louis, MO, USA) according to the manufacture's protocol. Equal amounts of protein were subjected to immunoblotting assay using specific antibodies: anti-poly (ADP-ribose) polymerase (PARP) antibody, anti-HIF-1α antibody, and anti-β-actin antibody.

Evaluation the injury of cells The apoptosis was determined by quantitative measurement of cytoplasmic histone-associated DNA fragments using an ELISA kit (Roche Diagnostics), or analysis the protein levels of cleaved PARP by immunoblotting assay with anti-PARP antibody.

FIGURES

Figure 3

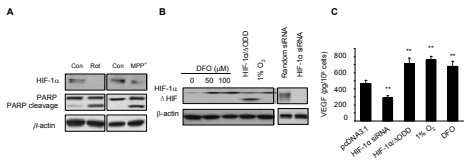


Figure 4

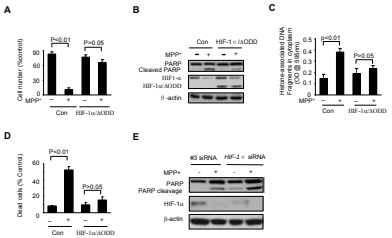
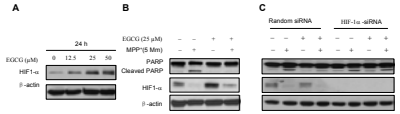


Figure 5



RESULTS

Regulation of HIF-1α in SH-SY5Y cells Our study showed that protein levels of HIF-1 α in SH-SY5Y cells were decreased after the cells were exposed to rotenone or MPP^+ (Figure 3A), indicating that mitochondrial inhibitors may enhance the degradation of HIF-1 α and lead to the reduction of HIF-1 α . On the other hand, the protein levels of HIF-1 α was increase in cells treated with iron chelator DFO, or cultured under hypoxic condition, or transfected with HIF-1 α / Δ ODD plasmid, whose molecular weight is lower than the normal one because of the deletion of ODD domain (Figure 3B). ELISA assay revealed that the protein levels of VEGF were increased in culture medium under the conditions that HIF-1 α was increased, whereas, in medium with HIF-1 α inhibition, the protein level of VEGF was decreased (Figure 3C), indicating that increased HIF-1 α may target on its target gene VEGF, and the function of HIF-1 α was largely maintained with the transfection by HIF-1 α /ΔODD plasmid.

Neuroprotection of HIF-1 α on MPP^+-induced neuronal injury Mitochondrial complex I inhibitor MPP^+ caused decrease in cell number (Figure 4A), increase of cleaved PARP and decrease of HIF-1a (Figure 4B), increase of histone-associated DNA fragments (Figure 4C), or the increase of dead cells (Figure 4D), all of which were diminished by enhanced expression of HIF-1 α (Figure 4A-4D). Suppression of HIF-1 α enhanced susceptibility of cells to MPP^+-induced apoptosis (Figure 4E).

Neuroprotection of EGCG *via* HIF-1 α EGCG treatment caused increase of HIF-1 α protein level in SH-SY5Y cells (Figure 5A). Pretreatment of cells with EGCG attenuated MPP^+-induced increase of cleaved PARP and recover the reduced protein levels of HIF-1 α caused by MPP^+ (Figure 5B), which effects were blocked when HIF-1 α gene was suppressed (Figure 5C).

LEGENDS

Figure 3. Protein levels of HIF-1 α under different conditions. (A) SH-SY5Y cells were exposed to mitochondrial complex I inhibitors rotenone or MPP^+ for 24 h. Total protein was subjected to immunoblotting assay with anti-HIF-1 α and anti-PARP antibodies. (B) SH-SY5Y cells were treated with DFO at 50 and 100 μM for 24 h, or cultured under hypoxia (1% O_2) for 24 h, or transfected with mutant HIF-1 α with ODD deletion expression plasmid (HIF-1 α /ΔODD) or transfected with HIF-1 α siRNA for 72 h using lipofectamine 2000. Total protein was isolated and subjected to HIF-1α immunoblotting assay. (C) The conditioned media from various cell cultures were used for detection VEGF level by ELISA kit. Data were expressed as Mean ± SD. P values (**: $p < 0.01$) were determined by one way ANOVA. Con = Control; Rot = Rotenone.

Figure 4. Role of HIF-1a in protecting against MPP^+-induced apoptosis. SH-SY5Y cells were transiently transfected with the HIF-1α/ΔODD for 24h and then exposed to MPP^+ for another 24 h. (A) The number of cells was counted and expressed as a percentage of vehicle control. The average was from three independent experiments. P values for the comparisons shown were determined by one way ANOVA. (B) The cell lysates were subjected to immunoblotting using the indicated antibodies. (C) The levels of histone-associated DNA fragmentation in the cytoplasm were quantified by ELISA assay. P values for the comparisons shown were determined by one way ANOVA. (D) After specific treatment as mentioned above, cells were stained with 1 μM calcein AM and 1.5 μM ethidium homodimer-1 for 20 minutes followed by the measurement of live or dead cells using live/dead assay kit. Dead cells were counted for three random microscopy sections. P values for the comparisons shown were determined by one way ANOVA. (E) SH-SY5Y cells were transfected with HIF-1α siRNA for 48 h followed by exposure to MPP^+ for additional 24 h. the protein levels of HIF-1α and cleaved PARP were determined by immunoblotting assay.

Figure 5. Role of pharmacologically induction of HIF-1α by EGCG in protecting against MPP^+-induced apoptosis (A) SH-SY5Y cells were treated with EGCG at various concentrations for 24 h and induction of HIF-1 α was determined by immunoblotting assay. (B) Cells were pre-treated with EGCG for 3 h followed by addition of MPP^+ for another 24 h. The protein levels of PARP were determined by immunoblotting assay (C). SH-SY5Y cells were transfected with HIF-1 α siRNA or radom siRNA for 48h, followed by exposure to MPP^+ for 24h with or without EGCG pretreatment. Total proteins were isolated and subjected to immunoblotting assay with antibodies indicated.

CONCLUSIONS

- We demonstrated that mitochondrial complex I inhibitors could cause apoptosis accompanied by the reduction in HIF-1 α protein level in SH-SY5Y cell. Over-expression of HIF-1 α by gene transfection or by treatment with compounds that may accumulate HIF-1 α , such as EGCG with properties of iron chelating, could protect against MPP^+-induced apoptosis. Whereas, suppression of HIF-1 α enhanced susceptibility of cells to MPP^+-induced apoptosis, or blocked neuroprtoective effects of EGCG on MPP^+.
- We conclude that HIF-1 α is an important gene for neuronal survival and that HIF-1 α activation might be a novel therapeutic strategy for PD. The mechanisms involved in the neuroprotection of HIF-1 α and its neuroprotective roles *in vivo* will be further explored.

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