·Research Article·

6-Hydroxydopamine up-regulates divalent metal transporter-1 and ferroportin-1 in C6 glioma cell lines

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Abstract
BACKGROUND: Previous studies have confirmed that 6-hydroxydopamine is capable to increase the expression of divalent metal transporter-1 and reduce the expression of ferroportin-1 in the neurons and microglia, which may lead to iron deposition in the substantia nigra after Parkinson's disease. However, it is unclear whether 6-hydroxydopamine can play diverse roles in astrocytes.
OBJECTIVE: To observe the effects of 6-hydroxydopamine on the expression of divalent metal transporter-1 and ferroportin-1 in rat C6 glioma cell lines.
METHODS: C6 glioma cell lines from rats were cultured in 10 μmol/L 6-hydroxydopamine for 24 hours. Then, protein expressions of divalent metal transporter-1 and ferroportiner-1 were measured by western blot method.
RESULTS AND CONCLUSION: The protein expressions of divalent metal transporter-1 and ferroportin-1 in C6 glioma cell lines were increased by 2.5 times ($P < 0.01$) and 1 time ($P < 0.05$), respectively, after treatment with 6-hydroxydopamine. These findings indicate that 6-hydroxydopamine can promote iron transport rate in astrocytes by increasing both divalent metal transporter-1 and ferroportin-1 expressions, and astrocytes has a different response to 6-hydroxydopamine from neurons and microglia.
Subject headings: Astrocytes; Oxidopamine; Iron; Membrane Transport Proteins

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INTRODUCTION

Parkinson's disease is a progressive neurodegenerative disorder characterized by the loss of dopaminergic neurons in the substantia nigra, giving rise to dopamine depletion in the striatum, which occurs mainly in the middle-aged and elderly with the clinical manifestations of akinesia, muscular rigidity, resting tremor and postural reflex disorder[1-3]. Although heredity, environment and oxidative stress play a certain role, the exact pathogenesis of Parkinson's disease is not fully understood.

Iron is one of the essential trace elements in the human body, which is widely involved in the body metabolic processes. The iron as an essential component involved in the synthesis of myelin sheath and neurotransmitters is particularly important for the brain. Existing evidence has shown that during the brain development, iron deficiency leads to irreversible behavioral and cognitive disorders in children, and excessive iron can cause damage to nerve cells, and thereby becomes one of the initial reasons for neurodegenerative diseases[4]. In this century, excessive iron-reduced damage to dopaminergic neurons in the substantia nigra-corpus striatum system is becoming an issue of attentions. Due to its cytotoxicity and its ability to promote hydroxyl radical production, iron has a non-negligible role in the pathogenesis of Parkinson's disease[5-7]. It is confirmed that iron accumulation is involved in the pathogenesis of Parkinson's disease. Selective deposition of irons exists in the substantia nigra of Parkinson's disease patients and animal models. We can

observe an increase in iron content in the substantia nigra of patients at early stage of Parkinson's disease, and moreover, the iron content is correlated with the progression of Parkinson's disease[8-9].

Studies have shown that an abnormal increase in iron content in the brain may be due to some iron transporters out of control. Divalent metal transporter-1 (DMT1) is the major iron into protein widely expressed in the brain[10-12], which has a similar structure to Nramp1 that consists of 12 transmembrane domains (**Figure 1**)[13]. DMT1 is widely expressed in the brain and highly expressed in the iron-enriched substantia nigra. Additionally, DMTI is also highly expressed in cells with demands for a lot of irons. Excessive iron deposition is likely to cause a series of pathological changes, and thereby destroy nerve cells, eventually leading to a variety of brain diseases, such as neurodegenerative diseases (including Parkinson's disease and Alzheimer's disease[14-15]. It has been reported that there is an excessive expression of DMTI in the substantia nigra neurons of Parkinson's disease patients, and DMT1 excessive expression may cause selective accumulation of irons and in turn result in an increase of reactive oxygen species, eventually leading to death of neurons[16-18].

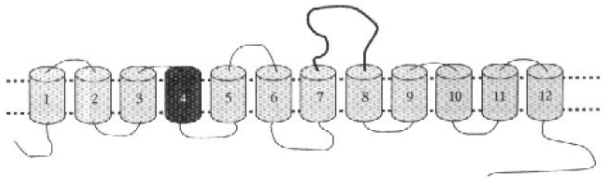

Figure 1 Predicted cartoon structure of divalent metal transporter-1[13]

Ferroportin-1 (FPN1) is currently the only known transmembrane protein for cellular iron efflux, which is also known as Fe-regulated transport protein 1 (IREG1) or metal transport protein 1 (MTP1)[19]. FPN1 consists of 571 amino acids and has at least 10 transmembrane domains (**Figure 2**), with a relative molecular mass of about 62 000[20]. Northernblot analysis shows that FPN1 is widely distributed in the body tissues, such as the small intestine, placenta, spleen, liver, kidney, heart, muscle, lung and brain. Brain studies in rats have shown that FPN1 is highly expressed in the hippocampus, cerebral cortex, cerebellum, thalamus and striatum, especially in the cortical pyramidal neurons and the apical dendrites. In addition, FPN1 is often found to highly express in the hippocampal pyramidal cells and granule cells as well as in the habenular nucleus. As previously reported, FPN1 is considered to be involved in iron deposition in the substantia nigra of Parkinson's disease by reducing the expression[21].

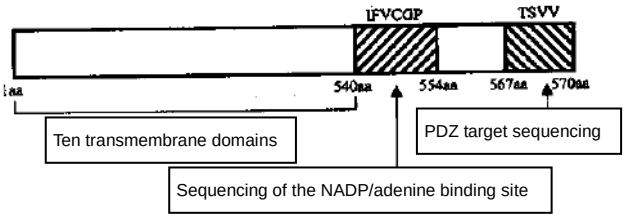

Figure 2 FPN1 Protein structure[20]

6-Hydroxydopamine (6-OHDA) is a widely used neurotoxic agent that can selectively damage dopaminergic neurons *in vivo* and *in vitro*. The toxicity of 6-OHDA is generated through reactive oxygen species-mediated oxidative damage to mitochondria[22]. In our previous studies, we have demonstrated that 6-OHDA can increase DMT1 but reduce FPN1 in neurons and microglia[23-24]. However, it is unclear whether 6-OHDA can play different roles in astrocytes. In this study, we aimed to investigate the changes of both DMT1 and FPN1 expressions in the C6 glioma cell lines after treatment with 10 μmol/L 6-OHDA for 24 hours.

MATERIALS AND METHODS

Design

In vitro cytological study.

Time and setting

Experiments were completed at the National Key Laboratory of Physiology, Qingdao University Medical School, China in July 2015.

Materials

Rat C6 glioma cell lines were provided by Shanghai Cell Bank of the Chinese Academy of Sciences. Unless otherwise stated, all chemicals were purchased from Sigma Chemical Co. (St. Louis., MO, USA). The primary antibodies against FPN1 and DMT1 were separately from the Sigma Chemical Co. and the Alpha Diagnostic (ADI, San Antonio, TX, USA). Dulbecco's modified Eagle's medium (DMEM) was from Gibco (Grand Island, NY, USA).

Methods

Cell cultures

C6 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 μg/mL streptomycin at 37 ℃, in a humid atmosphere of 5%

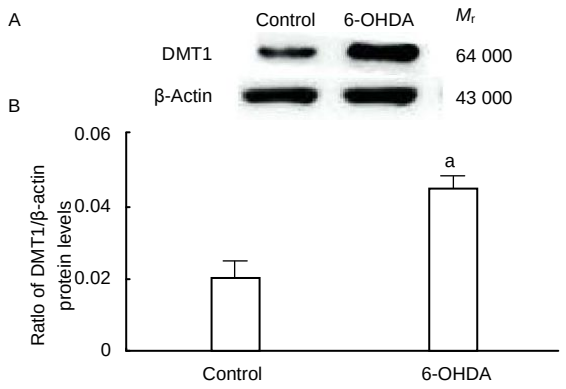

Figure 3 Protein level of DMT1 in 6-OHDA-treated C6 glioma cell lines

Note: (A) DMT1 expression was detected by western blot assay, which at 64 000 were higher in the 6-OHDA group than in the control group. (B) The relative protein level of DMT1 was significantly higher in the 6-OHDA group than the control group. [a]*P* < 0.01, *vs*. control group. DMT1: Divalent metal transporter-1; 6-OHDA: 6-hydroxydopamine.

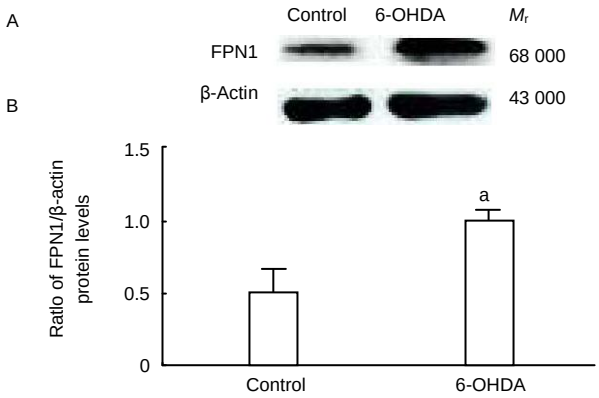

Figure 4 Protein level of FPN1 in 6-OHDA-treated C6 glioma cell lines

Note: (A) FPN1 expression was detected by western blot assay, which at 68 000 were higher in the 6-OHDA group than in the control group. (B) The relative protein level of FPN1 was significantly higher in the 6-OHDA group than the control group. [a]*P* < 0.05, *vs*. control group. FPN1: Ferroportin-1; 6-OHDA: 6-hydroxydopamine.

CO_2 and 95% air. For experiments, cells at 70%−80% confluence were sub-cultured and seeded at a density of 10^5 cells/cm^2.

Western blot analysis

Cells were treated with 10 μmol/L 6-OHDA for 24 hours and then collected after supernatant removal. After three washes with TBST, cells were digested directly on culture plates with RIPA lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% Nonidet-40, 0.5% sodium deoxycholate, 1 mmol/L ethylene diamine tetraacetie acid, 1 mmol/L phenylmethylsulfonyl fluoride) with protease inhibitors (pepstatin 1g/mL, aprotinin 1g/mL, leupeptin 1g/mL) for 30 minutes on ice. The insoluble material was removed by centrifugation (1 2 000 r/min, 20 minutes, 4 ℃). 30 μg total proteins were extracted and separated using 10% sodium dodecyl sulfate-polyacrylamide gels and then transferred to polyvinylidene fluoride membranes. Blots were probed with anti-DMT1 antibody (1:800, ADI) and anti-FPN1 antibody (1:800, Sigma). Blots were also probed with anti-β-actin monoclonal antibody (1:10 000, Sigma) as a loading control. Cross-reactivity was visualized using UVP gel imaging system and photographed. The DMT1 and FPN1 expressions were represented as the ratio of DMT1/β-actin and FPN1/β-actin. The experiment was repeated six times.

Main outcome measures

Changes in DMT1 and FPN1 expressions after 6-OHDA intervention.

Statistical analysis

Data are presented as mean±SEM and were statistically analyzed using SPSS 17.0 software. The intergroup comparisons were made by one-way analysis of variance and Tukey test. $P < 0.05$ and $P < 0.01$ were considered statistically or greatly statistically significant.

RESULTS

Expression of DMTI protein in 6-OHDA-treated C6 glioma cell lines

After treated with 10 μmol/L 6-OHDA for 24 hours, there was a 2.5-fold increase in DMT1 protein level in C6 glioma cell lines ($P < 0.01$; **Figure 3**).

Expression of FPN1 protein in 6-OHDA-treated C6 glioma cell lines

At 24 hours after 10 μmol/L 6-OHDA treatment, there was a 1-fold increase in FPN1 protein level in C6 glioma cell lines ($P < 0.05$; **Figure 4**).

DISCUSSION

Astrocytes as the major glial cell type in the central nervous system are widely distributed in the brain gray and white matters, and have multiple functions, including cell support during central nervous system development. Astrocytes participate in the various physiological or pathological processes, including the formation and maintenance of blood-brain barrier, neuronal development and synaptic remodeling, glutamate metabolism, and stable maintenance of extracellular K^+, and are generally accepted as a principle contributor for the uptake of a variety of nutrients such as transition

metals crossing the blood-brain barrier[25-28]. Additionally, astrocytes have a close relationship with central nervous system inflammation and immunoreactions[29-30]. DMT1 is mainly expressed in the end feet of astrocytes around the blood capillaries, indicating a major role of astrocytes in brain iron uptake. These cells have a strong ability to accumulate irons and have a higher tolerance to iron. Iron overloading can trigger neuronal cell and microglia death, and however, activate astrocyte proliferation[31]. Thus, astrocytes play an important role in regulating brain iron homeostasis and protecting other brain cells from iron-mediated oxidative stress.

6-OHDA, a norepinephrine homolog, can create oxidative stress through the production of free radicals and reactive oxygen to destroy dopaminergic neurons in the substantia nigra, and damage dopamine synthesis in the substantia nigra and the transport path to the striatum, thereby resulting in catecholamine and acetylcholine neurotransmitter imbalance and causing a series of symptom and pathological changes similar to human Parkinson's disease, such as unilateral rotation. 6-OHDA is now commonly used to establish animal models of Parkinson's disease and cell model of Parkinson's disease nerve gas, which is a kind of selective neurotoxins. Its toxicity on the one hand can happen outside the cells from oxidation, intracellular H_2O_2 and OH• indirect oxidative stress; on the other hand, 6-OHDA can directly enter into the cells by the dopamine transporter uptake and start to play a toxic role of oxidative stress[32-33]. In the 6-OHDA induced Parkinson' disease model and PD patients, glial cells in the substantia nigra pars compacta appear to have obvious responses. During the generation process, activated astrocytes and DA nerve endings produce a large number of "cross-talk", and reactive astrocytes in DA neuron degeneration play an important role.

Recent studies have shown that brain iron metabolism can cause a lot of brain diseases[34]. Iron is found a higher expression with the presence of oxidative stress in the brain basal ganglia and senile plaques of Alzheimer's disease and Parkinson's disease patients[35-36]. Increase in iron concentration in the brain can lead to iron oxidative stress and deficiency of antioxidant protection, which may be one reason for neuronal degenerative death. In recent years, studies have shown that the degree of oxidative damage in the brain tissue is directly related to the local brain iron, and iron-induced oxidative damage has been increasingly attracting attentions[37-38]. Accumulating evidence has demonstrated that the generation of reactive oxygen can be involved in the regulation of iron and subsequently regulate the iron-related proteins. Iron deposition in the substantia nigra is actively involved in the pathogenesis of Parkinson's disease.

Our previous studies have shown that increased expression of DMT1 and decreased expression of FPN1 can account for the iron deposition in both neurons and microglia with 6-OHDA treatment[23-24, 39]. In this study, we found that both DMT1 and FPN1 expressions were increased in C6 glioma cell lines treated with 10 μmol/L 6-OHDA for 24 hours. Experimental findings indicate that 6-OHDA promotes iron transport rate in astrocytes by increasing both DMT1 and FPN1 expressions, and astrocytes has a different response compared with neurons and microglia.

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神经毒素 6-OHDA 干预后星形胶质细胞铁转运蛋白表达的变化

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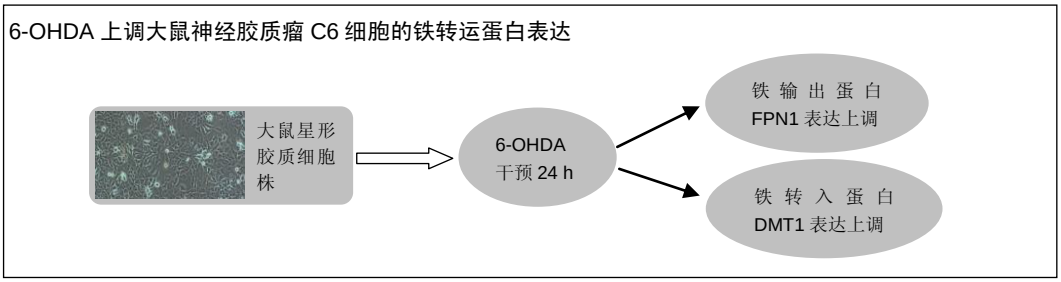
文章快速阅读：



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文题释义：

二价金属离子转运蛋白 1(DMT1)：是哺乳类跨膜铁转运蛋白，是主要的铁转入蛋白，广泛分布于人体各组织。主要功能是介导小肠上皮细胞的铁吸收以及参与铁从内吞小体移位到胞浆的过程，也参与其他二价金属如 Zn^{2+}、Mn^{2+}、Co^{2+}、Cd^{2+}、Cn^{2+}、Ni^{2+}和 Pb^{2+}的转运。在帕金森病患者的黑质发现 DMT1 表达异常增加，因而 DMT1 可能也与某些神经退行性疾病的形成有关。

膜铁转运蛋白 Ferroportin1(FPN1)：又称铁调节转运体(IREG1)或金属转运蛋白(MTP1)，是一种跨膜的铁输出蛋白，是唯一的细胞铁流出通道，在成熟的十二指肠绒毛上皮细胞基底面、脾和肝的巨噬细胞、胎盘的合体滋养层细胞等都有表达，对其研究可能对铁超载或铁缺乏疾病的诊断和治疗有重要的医学应用前景。

摘要

背景：前期的研究已经证实，在神经元和小胶质细胞中，神经毒素 6-OHDA 能够增加铁转入蛋白 DMT1 的表达，减少铁转出蛋白 FPN1 的表达，可能导致帕金森病黑质铁的沉积。然而，6-OHDA 能否在星形胶质细胞中发挥不同的作用尚不明确。

目的：观察 6-OHDA 作用于 C6 神经胶质瘤细胞(大鼠星形胶质细胞株)后，铁转运蛋白 DMT1 和 FPN1 表达变化。

方法：培养大鼠星形胶质细胞 C6 细胞，加入 10 μmol/L 6-OHDA 培养 24 h，Western blots 法检测 6-OHDA 干预后 DMT1 和 FPN1 蛋白表达。

结果与结论：用 10 μmol/L 6-OHDA 作用于大鼠 C6 星形胶质细胞 24 h 后，Western blots 检测结果显示 DMT1 蛋白表达升高了 2.5 倍($P < 0.01$)，FPN1 蛋白表达升高了 1 倍($P < 0.05$)。提示，6-OHDA 通过同时增加铁转运蛋白 DMT1 和 FPN1 表达促进铁运速率，星形胶质细胞对 6-OHDA 的反应与神经元和小胶质细胞不同。

关键词：

组织构建；组织工程；铁转运蛋白；DMT1；FPN1；星形胶质细胞；6-OHDA；帕金森病

主题词：

星形细胞；羟多巴胺；铁；膜转运蛋白质类

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