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The anti-aging protein klotho alleviates injury of nigrostriatal dopaminergic pathway in 6-hydroxydopamine rat model of Parkinson's disease: Involvement of PKA/CaMKII/CREB signaling

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ABSTRACT

Parkinson's disease (PD) is a prevalent movement disorder in the elderly. PD is hallmarked with progressive deterioration of mesencephalic dopaminergic neurons and development of debilitating motor and non-motor clinical symptoms. Klotho protein is the product of an aging-suppressor gene that its overexpression could protect neurons against oxidative injury. This study was undertaken to explore whether exogenous klotho could alleviate injury of nigrostriatal dopaminergic pathway in 6-hydroxydopamine (6-OHDA) rat model of PD. Intrastriatal 6-OHDA-lesioned rats were pretreated with klotho at a dose of 10 μg/rat. Results showed that klotho mitigates apomorphine-induced rotational behavior and reduces the latency to initiate and the total time in the narrow beam test. In addition, beneficial effect of klotho was attenuated following *i.c.v.* microinjection of protein kinase A (PKA) inhibitor H-89 and Ca(2 +)/calmodulin-dependent protein kinase II (CamKII) inhibitor KN-62. Additionally, klotho significantly lowered striatal levels of malondialdehyde (MDA), reactive oxygen species (ROS), glial fibrillary acid protein (GFAP), α synuclein, phospho-cAMP-response element binding protein (pCREB), and DNA fragmentation. Furthermore, klotho was capable to prevent degeneration of tyrosine hydroxylase (TH)-positive neurons within substantia nigra pars compacta (SNC). Collectively, these findings denote neuroprotective potential of exogenous klotho in 6-OHDA rat model of PD through alleviation of astrogliosis, apoptosis, and oxidative stress. It was also obtained that part of its protective effect is dependent on PKA/CaMKII/CREB signaling cascade.

1. Introduction

Parkinson's disease (PD) is a prevalent movement disorder in the elderly with an approximate incidence of 1.7% in individuals aged over 65 years (Zhang et al., 2005). The main pathologic hallmark of PD is the progressive degeneration of midbrain dopaminergic neurons and appearance of Lewy bodies (Beitz, 2014; Muangpaisan et al., 2011; Wirdefeldt et al., 2011). Lewy bodies are mainly composed of α-synuclein (Lázaro et al., in press). Clinical symptoms of PD comprise debilitating motor symptoms such as bradykinesia, resting tremor, rigidity, and stooping posture (Beitz, 2014; Rodriguez-Oroz et al., 2009). Although L-DOPA has been the most effective and gold standard therapy for PD, however, its long-term administration is associated with several detrimental motor and non-motor symptoms (Bastide et al., 2015; Sharma et al., 2015). Oxidative stress development, concurrent depression of antioxidant system (Ahmad et al., 2005; Kiasalari et al., 2016b), and inflammation (Gustot et al., 2015; Spittau, 2015) play pivotal roles in pathogenesis of PD. Protein kinase A (PKA)-dependent cascade plays a key role in brain homeostasis and control of inflammation and its dysfunction contributes to progression of neurodegenerative disorders like PD (Greggio et al., 2017). In addition, Ca (2 +)/calmodulin-dependent protein kinase II (CaMKII) plays an important role in the regulation of neuronal death and survival (Hajimohammadreza et al., 1995; Zhang et al., 2017b). cAMP-response element binding protein (CREB) is another intracellular signaling that its activation prevents dopaminergic cell death in PD (Ham et al., 2017). Innovative treatment strategies for PD, especially at its early stages, have gained paramount clinical attention (Beitz, 2014).

Klotho protein was first reported as a product of an aging-suppressor gene that is greatly expressed in kidney, brain, and parathyroid and pituitary glands (Kuro-o et al., 1997). Accumulating research evidence suggests that klotho could play vital roles as a neuroprotective agent

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and is able to promote myelination that is essential for healthy brain aging process, as reviewed before (Cararo-Lopes et al., 2017). A defect in klotho gene causes loss of hippocampal synapses, perturbations in axonal transport, neurofilaments changes, and lysosomes accumulation (Shiozaki et al., 2008). In addition, mesencephalic dopaminergic neurons show signs of neurodegeneration in klotho-insufficient mouse (Kosakai et al., 2011). Furthermore, klotho-deficient animals have higher levels of apoptosis signal-regulating kinase 1 (ASK1) with concurrent enhanced oxidative stress (Brobey et al., 2015a). Additionally, klotho could protect hippocampal neurons against amyloid and glutamate toxicity via activation of antioxidant enzymatic system (Abraham et al., 2016). Meanwhile, klotho overexpression protects dopaminergic neurons against oxidative injury (Brobey et al., 2015b). Klotho down-regulation is associated with aging-related inflammation and the development of early-stage chronic kidney disease (Zeng et al., 2016). Of interest, exogenous klotho increases anti-oxidative capacity via activation of nuclear factor erythroid 2–related factor 2 (Nrf2) signaling (Hsia et al., 2017). Hence, this study was undertaken to explore whether exogenous klotho could alleviate injury of nigrostriatal dopaminergic pathway in 6-hydroxydopamine rat model of PD.

2. Materials and methods

2.1. Animals and experimental procedure

Male adult Wistar rats (200–250 g; n = 72; obtained from breeding center of Iran University of Medical Sciences, Tehran, Iran) were housed in an animal facility with standard conditions (12/12 light/dark cycle, with lights on at 08:30 p.m.). The applied protocols for use and care of animals were approved by Ethics Committee of Iran University of Medical Sciences (Tehran, Iran) in 2015 and were in line with NIH regulations. Rats were randomly assigned to 6 equal-sized experimental groups: sham, klotho-treated sham (Sham + Klotho), lesion group (6-OHDA), lesion group receiving klotho (6-OHDA + Klotho), and two lesions groups receiving klotho and H-89 or KN-62 as specific inhibitors of protein kinase A (PKA) or Ca^{2+}/calmodulin-dependent kinase type II (CaMKII). For induction of PD model, the specific neurotoxin 6-OHDA was microinjected into the left striatum under deep anesthesia (ketamine at a dose of 80 mg/kg, i.p. and xylazine at a dose of 10 mg/kg, i.p.). For surgery, animals were fixed in a stereotaxic apparatus (Stoelting, USA) at coordinates 3 mm lateral and 0.2 mm anterior to bregma and ventrally 5 mm below the dura (Paxinos and Watson, 1986). The 6-OHDA group received 5 μl of cold normal saline consisting of 2.5 μg/μl of 6-OHDA-HCl (Sigma Aldrich, USA) and 0.2% ascorbate. The sham group received the same volume of intrastriatal normal saline-ascorbate solution. The klotho-treated 6-OHDA groups received 6-OHDA in addition to 5 μl of recombinant klotho *i.c.v.* (R & D Systems, Inc., USA) dissolved in cold normal saline at a dose of 10 μg/rat 30 min before 6-OHDA microinjection. Two lesions groups received klotho at the same dose and 2 μl of 0.5 μg/μl of H-89 (Tocris, USA) or KN-62 (Cayman Chemical, USA) as specific inhibitors PKA or CaMKII, 30 min before klotho microinjection. Dose of these inhibitors were chosen from an earlier study (de Araujo Herculano et al., 2011). The latter inhibitors were dissolved in DMSO with further dilutions in aCSF (containing 120 mM NaCl, 1.15 mM $CaCl_2$, 3 mM KCl, 0.8 mM $MgCl_2$, 27 mM $NaHCO_3$, and 0.33 mM NaH_2PO_4; pH was adjusted to 7.20) and were *i.c.v.* administered at coordinates 1.4 mm lateral and − 1 mm posterior to bregma and 3.6 mm below the dura.

2.2. Behavioral assessment

All behavioral experiments were conducted by a trained person blind to interventions and performed between 10:00 a.m. and 15:00 p.m. in a dimly-lighted and quiet arena.

2.2.1. Apomorphine-induced rotational test

The rotations were evaluated by a method as reported before (Sedaghat et al., 2014). Briefly, the animals were allowed to adapt for 15 min and full ipsilateral and contralateral turns were recorded after the injection of apomorphine hydrochloride (Sigma-Aldrich, USA; 2 mg/kg, i.p.) in a cylinder with a diameter of 33 cm and a height of 35 cm for 60 min. Finally, net number of rotations was calculated as positive rotations minus negative ones.

2.2.2. Elevated narrow beam test

The protocol for this test has been described before (Kiasalari et al., 2016a). The used apparatus composed of a wooden beam with a width of 4 cm, a length of 105 cm, and a height of 3 cm. It was placed at a height of 80 cm above the floor. Starting line was 20 cm away from its end. During the test, the rat was placed within the starting zone and upon animal freedom, time was recorded to reach starting line. This time shows the latency to initiate the task. The test finished when all four limbs were placed upon the finishing line at the other end of the beam. The cut-off time was 2 min. A fall was recorded as a maximum time. Each rat was tested four times on the beam with an interval of 5 min.

2.3. Oxidative stress assessment

One week post-surgery, left striatal and substantia nigra tissues (n = 7 for each group) were punched out and 5% homogenate was prepared in cold lysis buffer containing protease inhibitor cocktail (Sigma Aldrich, USA). The obtained supernatant was stored at − 70 °C.

2.3.1. Determination of MDA and ROS

For determination of MDA level (thiobarbituric acid reactive substances, TBARS), trichloroacetic acid and TBARS reagent were added to supernatant, then mixed and incubated at boiling water for 90 min. After cooling on ice, samples were centrifuged at 3000 × *g* for 5 min and the absorbance was read at 532 nm. The results were reported according to tetraethoxypropane standard curve (Kiasalari et al., 2016b).

ROS level was estimated with a non-fluorescent lipophilic dye, i.e. dichlorofluorescein diacetate, which is cleaved by intracellular esterase enzymes in the presence of ROS into 2,7-dichlorofluorescein that generates fluorescence (Arya et al., 2013; Tobon-Velasco et al., 2012). The fluorescence is directly proportional to the ROS level. Fluorescence was determined at 488 nm excitation and 525 nm emission. A standard curve was constructed using increasing concentrations of dichlorofluorescein incubated in parallel and results were expressed as ng of DCF (as ROS equivalent) formed.

Bradford method was applied for measurement of [protein] with bovine serum albumin as its standard (Bradford, 1976).

2.4. Determination of GFAP, α synuclein, and pCREB

The level of these parameters in the striatal and nigral supernatants was determined using sandwich enzyme-linked immunosorbent assay and commercial kit according to the manufacturer's instructions (for GFAP and α synuclein from Cloud-Clone Corp., USA and for pCREB from R & D Systems, Inc., USA). The absorbance of samples was read at 450 nm by Synergy HT microplate reader (BioTek, USA) and all values were reported according to related standard curves.

2.5. Determination of DNA fragmentation

DNA fragmentation as a reliable index of apoptosis was determined using the Cell Death Detection ELISA kit (Roche Diagnostics, Germany) as described before (Afshin-Majd et al., 2015; Morroni et al., 2013) by a microplate reader (BioTek, USA) to obtain OD.