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Transduced PEP-1-PON1 proteins regulate microglial activation and dopaminergic neuronal death in a Parkinson's disease model

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ABSTRACT

Parkinson's disease (PD) is an oxidative stress-mediated neurodegenerative disorder caused by selective dopaminergic neuronal death in the midbrain substantia nigra. Paraoxonase 1 (PON1) is a potent inhibitor of low-density lipoprotein (LDL) and high-density lipoprotein (HDL) against oxidation by destroying biologically active phospholipids with potential protective effects against oxidative stress-induced inflammatory disorders. In a previous study, we constructed protein transduction domain (PTD) fusion PEP-1-PON1 protein to transduce PON1 into cells and tissue. In this study, we examined the role of transduced PEP-1-PON1 protein in repressing oxidative stress-mediated inflammatory response in microglial BV2 cells after exposure to lipopolysaccharide (LPS). Moreover, we identified the functions of transduced PEP-1-PON1 proteins which include, mitigating mitochondrial damage, decreasing reactive oxidative species (ROS) production, matrix metalloproteinase-9 (MMP-9) expression and protecting against 1-methyl-4-phenylpyridinium (MPP^+)-induced neurotoxicity in SH-SY5Y cells. Furthermore, transduced PEP-1-PON1 protein reduced MMP-9 expression and protected against dopaminergic neuronal cell death in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice model. Taken together, these results suggest a promising therapeutic application of PEP-1-PON1 proteins against PD and other inflammation and oxidative stress-related neuronal diseases.

1. Introduction

Reactive oxygen species (ROS) serve multiple functions in a number of cellular processes, including the physiological phenomenon of embryonic development, aging, inflammatory disorders, and neurodegenerative diseases [1]. At low levels ROS act as cAMP second messengers to promote cell proliferation and cell survival [2], whereas, excessive ROS induces inflammatory response, cellular damage, and oxidative stress leading to cell death. A major organelle of endogenous ROS production is mitochondria from complex I and III of the electron transport chain. Other sources of ROS generation include membrane-associated NADPH oxidase, cytochrome *c* oxidase, and xanthine oxidase. The biological damage done by ROS and reactive nitrogen species (RNS) is called oxidative stress and nitrosative stress. One of the biomarkers of oxidative stress is lipid peroxidation, as fatty acids in the cell membrane are vulnerable to ROS attack. The oxidative modification of low-density lipids (Ox-LDL) accumulated in macrophages represent an inflammatory mediator that stimulates the inflammatory response associated with inflammatory disorders [3–5].

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Parkinson's disease (PD) is a major neurodegenerative disorder that affects at least four million people. PD is characterized by progressive loss of dopamine-producing neurons in the substantia nigra [6]. Elevated ROS production in the midbrain is mainly due to oxidative stress-induced inflammation regulated by microglial activation [7]. 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a neurotoxin that causes permanent symptoms of PD [8]. MPTP is metabolized to 1-methyl-4-phenylpyridinium (MPP^+) by MAO-B enzymes in microglial cells. MPP^+ is taken up by dopaminergic neurons via the dopamine transporter and induces oxidative stress, leading to mitochondrial damage and dopaminergic neuronal death [9].

In addition, lipopolysaccharide (LPS) is recognized by TLR4 on microglia that might be involved in neuronal death by activating microglial cells and pro-inflammatory cytokines thus causing subsequent neurotoxicity [10]. Activation of microglia can generate both intracellular and extracellular ROS. High levels of intracellular ROS promote uncontrolled inflammatory response and may result in the production of neurotoxic factors that contribute to cell death. Interestingly, extracellular ROS is neurotoxic to dopaminergic neurons and increases neuroinflammatory cytokines, suggesting that the regulation of ROS production is the most critical element of PD studies of this nature [11].

Paraoxonase 1 (PON1) is a member of the paraoxonase family of proteins and is ubiquitously expressed in many tissues, but highly expressed in the liver. Endogenous PON1 was transduced into cells and attached to HDL in the plasma membrane and served to prevent LDL and HDL oxidation. Concentration of PON1 proteins in serum affected inflammatory response and levels of oxidized-LDL (Ox-LDL) associated with various human diseases, including type 2 diabetes, inflammatory bowel diseases and Parkinson's disease [12–14]. Previous studies have reported that PON1 gene deficient animals demonstrated increased oxidative stress. In contrast, mice with overexpressed PON1 demonstrated decreased lipid hydroperoxide production. In addition, injection of human PON1 into *apo*E-knockout mice promoted progressive arteriosclerosis [15–17]. These features of PON1 proteins clearly support the notion that PON1 proteins are sufficient to restrain production of ROS and inflammation. Given that, we sought to deliver PON1 proteins into cells. Therefore we designed and generated cell permeable peptides (PEP-1) conjugated with PON1 proteins. Protein transduction domains (PTDs) are basic peptide sequences present in proteins and have the ability to deliver proteins into cells. Among PTDs, PEP-1 peptides, consisting of three domains, have greater efficiency in delivering target proteins, regardless of size, into cells [18].

In this study, we provide evidence that exogenous PEP-1-PON1 proteins inhibit oxidative stress-induced inflammation in microglia and suppress mitochondrial dysfunction and neurotoxicity in neurons. Furthermore, efficient delivery of exogenous PEP-1-PON1 proteins is internalized in PD-induced mice suggesting that PEP-1-PON1 has potential to be developed into an effective and useful therapeutic agent against oxidative stress induced diseases including PD.

2. Materials and methods

2.1. Cell lines and reagents

Mouse microglial BV2 and human neuroblastoma SH-SY5Y cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in a 5% CO_2 incubator.

LPS, Methyl-4-phenylpyridinium (MPP^+), 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and dichlorofluorescein diacetate (DCF-DA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodine (JC-1) was purchased from Cayman Chemical (Ann Arbor, MI, USA). PEP-1 peptide was synthesized from PEPTRON (Daejeon, Korea). Enhanced chemiluminescence agent was purchased from Amersham (Franklin Lakes, NJ, USA). The following antibodies were used for WB or immunostaining analysis: Akt, p-Akt, ERK, p-ERK, JNK, p-JNK, p38, p-p38, iNOS, beta actin, caspase 3, c-caspase 3, Bcl-2, p65, p-p65, IκBα, p-IκBα, Bax, and MMP-9. They were obtained from Cell Signaling Technology (Beverly, MA, USA). COX2, His-tagged, Tyrosine Hydroxylase (TH), and TNF-alpha were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Animal study

Eight-week-old, male C57BL/6 mice were acquired from the Hallym University Experimental Animal Center. They were housed at 23 °C and humidity of 60%. They were exposed to regulated 12 h cycles of light and dark and were given ad libitum access to food and water. Procedures for the care of animals conformed to the Guide for the Care and Use of Laboratory Animals of the National Veterinary Research and Quarantine Service of Korea and approved by Hallym Medical Center Institutional Animal Care and Use Committee.

2.3. Expression and purification of PEP-1-PON1 proteins

Engineering of the PEP-1-PON1 conjugated expression vector was described previously [19]. Briefly, human PON1 cDNA was obtained via PCR amplification with the following primers: sense primer, 5′-CTCGAGGCGAAGCTGATTGCG-3′, anti-sense primer, 5′-GGATCCTTAGAGCTCACAGTAAAGAGC-3′. The PCR products were subcloned in a TA-cloning vector and ligated into a PEP-1 conjugated expression vector with an N terminal His_6-tagged protein. A control PON1 expression vector was also constructed without the PEP-1 domain. Transformed BL21(DE3) *Escherichia coli* cells containing one PEP-1-PON1 construct were inoculated in 100 ml of LB media containing 100 μg/ml ampicillin and 30 μg/ml at 37 °C to $OD_{600\ nm}$ of 0.5–1.0. 0.5 mM final concentration of IPTG was added and the temperature was adjust to 30 °C for 5–6 h. Clear lysates were prepared by sonication at 4 °C in a binding buffer (5 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9) and were centrifuged and loaded into an Ni^{2+}-nitrilotriacetic acid Sepharose affinity column (Qiagen, Valencia, CA, USA). After washing the column with 10 volumes of binding buffer, they were washed with 6 volumes of a washing buffer (35 mM imidazole, 500 mM NaCl, and 20 mM Tris–HCl, pH 7.9). 6xHis tagged PEP-1-PON1 and control PON1 proteins were eluted with an elution buffer (0.5 M imidazole, 500 mM NaCl, 20 mM Tris–HCl, pH 7.9). The eluted PEP-1-PON1 proteins were desalted with PD10 column chromatography (Amersham, Braunschweig, Germany).

2.4. Transduction of PEP-1-PON1 proteins into BV2 cells, SH-SY5Y cells, and mice

For the *in vitro* transduction experiment, microglial BV2 cells and neuroblastoma SH-SY5Y cells were maintained in DMEM with 10% FBS, 4 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin in 5% CO_2 at 37 °C. Microglial BV2 cells and human neuroblastoma SH-SY5Y cells were transduced with PEP-1-PON1 proteins at different concentrations (0.1–1 μM) for 1 h or various periods of time (10–120 min) at 1 μM of PEP-1-PON1. Control PON1 or PEP-1 peptides were used as controls. Then, cells were treated with trypsin-EDTA and washed with phosphate-buffered saline (PBS). All cell extracts were prepared and measured by Western blotting [19].