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LACTOFERRIN FROM BOVINE COLOSTRUM REGULATES PROLYL HYDROXYLASE 2 ACTIVITY AND PREVENTS PRION PROTEIN-MEDIATED NEURONAL CELL DAMAGE VIA CELLULAR PRION PROTEIN

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Abstract—Prion disorders are associated with the conversion of normal cellular prion protein (PrPc) to the abnormal scrapie isoform of prion protein (PrPsc). Recent studies have shown that expression of normal PrPc is regulated by hypoxia-inducible factor-1 alpha (HIF-1α), and that lactoferrin increases full-length PrPc on the cell surface. Lactoferrin is an 80-kDa iron-binding glycoprotein with various biological activities, including iron-chelating ability. HIF-1α and the associated ubiquitin–proteasome pathway are regulated by HIF prolyl-hydroxylases 2 (PHD2). We hypothesized that lactoferrin regulates PHD2 expression and enzymatic activity, and the PHD2 regulation promotes HIF-1α stability and prevention of neuronal cell death mediated by prion protein (PrP) residues (106–126). Lactoferrin prevented PrP (106–126)-induced neurotoxicity by the induction of PrPc expression *via* promoting HIF-1α stability in neuronal cells. Our results demonstrated that lactoferrin prevented PrP (106–126)-induced neurotoxicity *via* the up-regulation of HIF-1α stability determined by PHD2 expression and enzymatic activity. These findings suggest that possible therapies such as PHD2 inhibition, or promotion of lactoferrin secretion, may have clinical benefits in neurodegenerative diseases, including prion disease.

Key words: lactoferrin, PrP (106–126), prion disease, hypoxia inducible factor 1 alpha (HIF-1α), HIF prolyl hydroxylase2 (PHD2).

INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are a group of rare, progressive diseases of the brain affecting mammals, including humans and domestic animals (Sakudo and Ikuta, 2009). Prion disorders are associated with the conversion of normal cellular prion protein (PrPc) to the abnormal, scrapie isoform of the prion protein (PrPsc) (O'Donovan et al., 2001). Compared with PrPc, PrPsc is resistant to proteases and shows a strong propensity toward accumulating as insoluble fibrils that interrupt normal neuronal function (Sakudo and Ikuta, 2009).

A synthetic peptide PrP (106–126) has been found to induce apoptosis in primary murine cerebellar cultures (Brown et al., 1996), primary rat hippocampal cultures (Forloni et al., 1993), and the human neuroblastoma cell line SH-SY5Y (O'Donovan et al., 2001). Therefore, prion protein (PrP; 106–126) is a recognized model for studying the characteristics of PrPsc (O'Donovan et al., 2001).

Hypoxia-inducible factor-1 (HIF-1) is a heterodimeric transcription factor composed of a constitutively expressed β-subunit (HIF-1β) and an oxygen-regulated α-subunit (Ziello et al., 2007). Alpha-subunits consist of three structurally similar subunits, HIF-1α, HIF-2α, and HIF-3α. HIF-1 is a key regulator of metabolic acclimatization to hypoxia (Maloyan et al., 2005). Previous studies have shown that hypoxia protected against PrP (106–126)-induced neuronal cell death *via* activation of HIF-1α (Seo et al., 2010; Jeong et al., 2012). Furthermore, HIF-1α regulated PrPc expression in neuronal cells (Jeong et al., 2012). Under hypoxic conditions, there is increased HIF-1α expression (Coimbra et al., 2004) as well as HIF-1α stability (Berra et al., 2003). By contrast, under normoxic conditions, HIF-1α is constitutively synthesized and degraded by the ubiquitin–proteasome pathway. Proteosomal degradation is initiated by hydroxylation of the two proline residues (P402 and P564 in HIF-1α) in its oxygen degradation domain (ODD) by the HIF prolyl hydroxylase domain proteins (PHDs) (Ke and Costa, 2006).

Proteins level of HIF-1α are regulated by the "oxygen sensing" activity of PHDs (Ke and Costa, 2006). PHDs comprise three isoforms: PHD1 (also known as EGLN2 or HPH3), PHD2 (also known as EGLN1 or HPH2), PHD3 (also known as EGLN3 or HPH1), all shown to

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Abbreviations: CHX, cycloheximide; DFO, deferoxamine; DMOG, dimethyloxalylglycine; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIF-1α, hypoxia-inducible factor-1 alpha; ODD, oxygen degradation domain; PBS, phosphate-buffered saline; PHD, prolyl-hydroxylases 2; PrP, prion protein; PrPc, normal cellular prion protein; PrPsc, abnormal scrapie isoform of prion protein; qRT-PCR, quantitative real-time polymerase chain reaction; shHIF-1α, shRNA plasmids for HIF-1α; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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hydroxylate the key proline residues of HIF-1α *in vitro* (Berra et al., 2003).

In addition, PHD inhibitors such as deferoxamine (DFO), activate HIF-1α and prevent neuronal cell death (Baek et al., 2011). Recent major advances have shown that HIF-1α-associated ubiquitin-proteasome pathway, is regulated by HIF prolyl-hydroxylases 2 (PHD2) (Berra et al., 2003). Furthermore, PHD2 is generally considered to be the regulator of HIF-1α in human cells (Jeong et al., 2012). Under normoxic conditions, inhibition of PHD2 but not PHD1 and PHD3 expression was shown to stabilize HIF-1α protein levels (Berra et al., 2003).

Lactoferrin is an 80-kDa iron-binding glycoprotein found in colostrum, milk, and other mucosal secretions such as saliva and tears (Bennett and Kokocinski, 1978; Kijlstra et al., 1983; Sanchez et al., 1988; Caccavo et al., 2002; Fine et al., 2002). It is synthesized by glandular epithelial cells and neutrophils (Gonzalez-Chavez et al., 2009; Alexander et al., 2012). Lactoferrin has various biological functions such as regulation of neutrophil apoptosis, antimicrobial ability, anti-inflammatory effect, and iron chelation ability, and also has anti-prion activities *via* maintaining PrPc on the cell-surface (Iwamaru et al., 2008). Recent studies revealed that bovine lactoferrin and apo-lactoferrin may act as a normoxic mimetic of hypoxia through stabilizing HIF-1α (Zakharova et al., 2012; Nguyen et al., 2014).

In this paper, we hypothesized that lactoferrin-mediated inhibition of PHD2 enzymatic activity prevents PrP (106–126)-induced neuronal cell death. To test this hypothesis, we investigated both, the effect of lactoferrin on PHD2 enzymatic activity as well as interrelation between lactoferrin and HIF-1α on PrP (106–126)-induced neuronal cell death. Our results showed that lactoferrin regulated PHD2 enzymatic activity and thus inhibited HIF-1α degradation and increased HIF-1α stability in normoxic conditions. Accordingly, the findings from the current study indicated that lactoferrin prevented PrP (106–126)-induced neuronal cell death *via* increased HIF-1α stability through inhibition of PHD2 enzymatic activity.

EXPERIMENTAL PROCEDURES

Cell culture

The SH-SY5Y human neuroblastoma cell line was obtained from the American Type Culture collection (ATCC, Rockville, MD, USA). Murine neuronal cell lines, ZW 13-2 and Zpl 3-4 established from the hippocampus of ICR ($Prnp^{+/+}$) and Zürich I $Prnp^{-/-}$ mice, respectively, were provided by Professor Yong-Sun Kim (Hallym University, Chuncheon, Gangwon-do, South Korea). SH-SY5Y cells were cultured in Minimum Essential Medium (MEM; Invitrogen-Gibco, Grand Island, NY, USA), whereas ZW 13-2 and Zpl 3-4 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; HyClone, Logan, UT, USA) that contained 10% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA) and Penicillin–Streptomycin (both 100 units/ml) in a humidified incubator maintained at 37 °C and 5% CO_2.

Construction of HIF-1α *short hairpin (sh)* RNA plasmid

The shRNA against HIF-1α was provided by Dr. Yong-Nyun Kim (National Cancer Research UK, Goyang, Gyeonggi-do, South Korea). The shRNA plasmids for HIF-1α (shHIF-1α) were constructed in the lentiviral vector pL-UGIP. The shRNA for HIF-1α was obtained with oligonucleotide forward 5′-CTGATGACCAGCA ACTTGA-3′ and reverse 5′-TCAAGTTGCTGGTCAT CAG-3′ sequences. SH-SY5Y cells were transfected with shHIF-1α, and stable transfectants were selected in puromycin after 24-h recovery in standard growth medium. SH-SY5Y cells transfected with mock vector (shMOCK) were used as controls.

Reagents

Lactoferrin isolated from cow colostrum, dimethyloxalylglycine (DMOG) and DFO and doxorubicin were purchased from Sigma. Cycloheximide (CHX) and deferiprone were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

PrP (106–126) treatment

Synthetic PrP (106–126) (sequence, Lys-Thr-Asn-Met-Lys-His-Met-Ala-Gly-Ala-Ala-Ala-Ala-Gly-Ala-Val-Val-Gly-Gly-Leu-Gly) was synthesized by Peptron (Daejon, South Korea). The peptide was purified up to 91% of purity using HPLC and dissolved in sterile dimethyl sulfoxide (DMSO) at a concentration of 10 mM and stored at −80 °C.

Western blot

SH-SY5Y cells were lysed in a buffer containing 25 mM HEPES; pH 7.4, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 0.1 mM dithiothreitol (DTT), and protease inhibitor mixture. Proteins were electrophoretically resolved by 10–15% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotting was performed as previously described. Equal amounts of lysate protein were electrophoretically resolved and transferred to a nitrocellulose membrane. Immunoreactivity was detected through sequential incubation with primary antibody, horseradish peroxidase-conjugated secondary antibody and enhanced chemiluminescence reagents. Primary antibodies used for immunoblotting were HIF-1α (Pierce Biotechnology, IL, USA), HO-HIF-1α (Cell Signaling Technology, Boston, MA, USA), PHD2 (Abcam, Cambridge, MA, USA), and PrPc (Millipore, MA, USA), β-actin (Santa Cruz Biotech).

Annexin V assay

Apoptosis was assessed by a commercial annexin V assay (Santa Cruz Biotech.) according to the manufacture's protocol. Annexin V content was determined by measuring fluorescence at an excitation wavelength of 488 nm and emission wavelengths of