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Aβ-affected pathogenic induction of S-nitrosylation of OGT and identification of Cys-NO linkage triplet

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ABSTRACT

Mechanistic link of protein hypo-O-GlcNAcylation to the pathogenesis of Alzheimer's disease (AD) remains unclear. Here, we found that S-nitrosylation of O-linked *N*-acetylglucosaminyltransferase (SNO-OGT) was induced by β-amyloid peptide (Aβ) exposure to SK-N-MC and SK-N-SH human neuroblastoma cells. Subsequently, Aβ-induced SNO-OGT led to protein hypo-O-GlcNAcylation globally including tau hypo-O-GlcNAcylation. Our results support that underlying mechanism for induction of SNO-OGT comprises the concerted action of Aβ-triggered Ca^{2+} entry into cells and nNOS-catalyzed NO production. Intriguingly, OGT was found to be associated with nNOS and its association was enhanced during Aβ treatment. In parallel with SNO-OGT-mediated tau hypo-O-GlcNAcylation, Aβ led to SNO-Akt-mediated GSK3β activation for tau phosphorylation, suggesting that tau hyperphosphorylation is established by synergistic connection between SNO-OGT and GSK3β activation. We also observed that Aβ-neurotoxicity including both reactive oxygen species (ROS) production and cell death was amplified with DON treatment, whereas it was restored by PUGNAc treatment, $GlcNH_2$ treatment or OGT overexpression. Early time-course Aβ-monitoring assay revealed that premaintained hyper-O-GlcNAcylation inside cells blocked not only Aβ-triggered Ca^{2+} entry into cells but also induction of SNO-OGT and SNO-Akt. Together, these findings suggest that induction of SNO-OGT by Aβ exposure is a pathogenic mechanism to cause cellular hypo-O-GlcNAcylation by which Aβ neurotoxicity is executed, and conversely, hyper-O-GlcNAcylation within cells can defend against Aβ neurotoxicity. Furthermore, our Cys mapping demonstrates that cysteine-nitric oxide (Cys-NO) linkages in SNO-OGT occur at triple Cys845, Cys921, and Cys965 residues in C-terminal catalytic domain (C-CAT), suggesting that Cys-NO linkage triplet in SNO-OGT is associated with null OGT activity.

1. Introduction

Alzheimer's disease (AD) is the most common amyloid neurodisease represented by senile plaques containing extracellular deposition of β-amyloid peptide (Aβ), intracellular neurofibrillary tangles (NFT) of tau proteins, and neuronal loss [1–6]. Early onset of both sporadic and familial AD pathogenesis has been progressed primarily by neurotoxic impacts of soluble oligomers of Aβ [7–9]. Proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretase generates multiple forms of 39–42 amino acids long Aβ, including $Aβ_{1-40}$ and $Aβ_{1-42}$ as major types [10–12]. It has been demonstrated that soluble oligomers of Aβ ranging from 2–3-mers to 12–24-mers are upstream players contributed to neurotoxicity by causing dysregulation of calcium homeostasis in neuronal cells [13–15]. Recent studies have shown that Aβ oligomers-mediated increase of cytosolic calcium ($[Ca^{2+}]_i$) propagates downstream neurotoxic effects, such as nitric oxide (NO) production, mitochondrial dysfunction, ROS accumulation, and apoptotic neuronal death [16–20].

Nitric oxide (NO) is a neurotransmitter which is produced in glia and neurons by nitric oxide synthase (NOS) and protein S-nitrosylation mediated by NOS-catalyzed NO has been shown to play important roles in associated with pathophysiology in central nervous systems [21–23]. NO stress in AD can be derived from iNOS (inducible NOS)- and nNOS (neuronal NOS)-catalyzed NO during glial cell activation, neuronal inflammation, and excitatory stimulation of NMDA-R or glutamate receptors [24]. Molecular targets of nNOS-catalyzed NO have been revealed by proteomic approach of S-nitrosylation in neuronal system [25]. It has been reported that S-nitrosylation of Drp-1 facilitates mitochondrial fission and neuronal injury in AD [26] and activated Cdk5 by S-nitrosylation has been shown to contribute to dendritic synaptic loss [27]. Recently, it has been shown that aberrantly S-nitrosylated proteins play pathogenic roles in AD and Parkinson's diseases (PD) [28].

Ser/Thr-linked O-GlcNAcylation of numerous cytosolic and nuclear proteins catalyzed by O-linked *N*-acetylglucosaminyltransferase (OGT) is widely known to be important for modulation of protein's functionality in neucleocytoplasm [29–33]. Glucose metabolism through hexosamine biosynthetic pathway (HBP) is indispensable for

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protein O-GlcNAcylation [34]. Hypo-glucose uptake and hypo-glucose metabolism have been observed in AD patients [35, 36], implying a possible link between hypo-glucose metabolism and neurodegeneration of AD. It has been shown that reduced O-GlcNAcylation is revealed in brain tissue extracts of AD patients [37, 38], however, underlying mechanism is not fully understood. Therefore, Aβ-mediated neurotoxic pathway in relation with protein O-GlcNAcylation needs to be solved detailedly in neuronal cell system [39, 40].

Recently, S-nitrosylation of OGT (SNO-OGT) and denitrosylation of OGT (DeNO-OGT) have been initially described [41]. In the present study, we further investigate the mechanistic link of SNO-OGT to AD pathogenesis in Aβ-affected neuronal cells. Here, we provide evidences that Aβ-affected induction of SNO-OGT provokes cellular hypo-O-GlcNAcylation including tau hypo-O-GlcNAcylation, by which Aβ neurotoxicity can be executed. Furthermore, our Cys mapping with biotin-switch assay demonstrates that triple Cys845, Cys921, and Cys965 in C-terminal catalytic domain (C-CAT) are identified to be linked with NO in SNO-OGT.

2. Materials and methods

2.1. Reagents

NMDA, Memantine, Streptavidin, and Fura 2-AM were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Calcium ionophore A23187, hexafluoroisopropanol (1,1,1,3,3,3-hexafluoro-2-propanol), 2′,7′-Dichlorofluorescein diacetate, Anti-flag antibody (M2), Anti-flag M2 affinity gel, Anti-OGT antibody (DM-17), Greiss reagents, DON (6-diazo-5-oxo-L-norleucine), L-MMA(N-methyl-L-arginine), Ascorbate, Neocuproine, and MMTS (S-methyl methanethiosulfonate) including all other chemicals for biotin-switch assay were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Biotin-HPDP (EZ-Link), NeutrAvidin-agarose resins, and phosphatase inhibitor cocktails were obtained from Thermo (Waltham, MA, USA). PUGNAc (O-(2-Acetamido-2-deoxy-D-glucopyranosylidene)amino-N-phenyl carbamate) was obtained from Toronto Research Canada (Toronto, Canada). ELISA 96 well plate (EIA/RIA) was obtained from Corning Co. (New York, NY). β-Amyloid peptide (Aβ(25–35) and Aβ(1–42) peptide) were purchased from ANASPEC (Fremont, OH). Protease inhibitor cocktail was purchased from Merck (Frankfurter, Germany). Anti-β-actin antibody was obtained from Cell Signaling (Danvers, MA) and Anti-O-GlcNAc antibody (RL2) was purchased from Abcam (Cambridge, MA). Antibodies for Akt, pS473-Akt, GSK3, pS9-GSK3, Tau, phospho-Ser, phospho-Thr, His_6-tag (G-18), and MBP-tag (N-17) were obtained from SantaCruz Biotechnology (Dallas, Texas).

2.2. Cell culture and transfection

Human neuroblastoma cell line of SK-N-MC and SK-N-SH cells, and HEK 293 T cells were obtained from ATCC (Rockville, MD). Cells were maintained in DMEM (GIBCO/BRL) media containing 10% fetal bovine serum (Hyclone, UT) supplemented with 100 U/ml penicillin and 100 U/ml streptomycin antibiotics, and were grown as monolayers at 37 °C in a humidified incubator with 5% CO_2 in air. Full-length cDNA encoding human wt OGT and mutant OGT constructed in pCMV-Tag 2B were transfected into SK-N-MC cells and HEK 293 T cells using Lipofectamine (Gibco-BRL) according to the manufacturer's instructions. Transient and stable single cells were selected with G418 at 400 μg/ml and OGT expression was assessed by RT-PCR, and further verified by immunoblotting using Flag-tag-specific M2 antibody.

2.3. DNA constructions and site-directed mutagenesis

Total RNA was purified from SK-N-MC cells by easy-spinTM total RNA Extraction Kit (iNtRON) and cDNA was synthesized by AccuScriptTM High Fidelity 1st-strand cDNA Synthesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Human ncOGT sequence from SK-N-MC cells was prepared by RT-PCR amplification using exTaq (Takara-Shuzo). Full-length OGT (wild type, 1-1046aa), TPR (1-569aa), and CAT (570-1046aa) were constructed in Flag™-tag mammalian expression vector (pCMV-Tag2B, Stratagene) and in His_6-tag bacterial expression vector (pET28a(+)) as described [41]. Mutation of Cys residues in CAT domain of OGT was performed by PCR amplification with mutagenesis-specific primers using a Quick Change Kit (Stratagene) according to the manufacturer's instructions. Mutated plasmid DNA was transformed to XL-Blue *Escherichia coli* and mutation of Cys residues was verified by DNA sequencing.

2.4. Cysteine mutagenesis-specific primer sequences

Mutation of Cys residues in CAT domain of OGT was performed by PCR amplification with mutagenesis-specific primers (9 sets of fP: forward primer and rP: reverse primer) using a Quick Change Kit (Stratagene) according to the manufacturer's instructions. Mutated OGT-plasmid DNA was transformed to XL-Blue *E. coli* and mutagenesis of Cys residues was verified by DNA sequencing.

Cys531.
fP:5′-gagaggcacggcaacctg**agc**ttagataagattaatg-3′/rP:5′-cattaatcttatctaa**gct**caggttgccgtgcctctc-3′.

Cys590.
fP:5′-gataaatttgaggtgttc**agt**tatgccctgagcccag-3′/rP:5′-ctgggctcagggcata**act**gaacacctcaaatttatc-3′.

Cys758.
fP:5′-gaaaattgtcaagatgaag**agt**cctgatggaggagac3′/rP:5′-gtctcctccatcagg**act**cttcatcttgacaattttc-3′.

Cys620.
fP:5′-gatctttctcagattcca**agc**aatggaaaagcagctg-3′/rP:5′-cagctgcttttccatt**gct**tggaatctgagaaagatc-3′.

Cys845.
fP:5′-gaagatgccatcgtatac**agt**aactttaatcagttg-3′/rP:5′-caactgattaaagtt**act**gtatacgatggcatcttc-3′.

Cys921.
fP:5′-gccagctggctgatgtc**agc**ttggacactccactc-3′/rP: 5′-gagtggagtgtccaa**gct**gacatcagccagctggc-3′.

Cys927.
fP:5′-ctgcttggacactccactc**agt**aatgggcacaccacagg-3′/rP: 5′-cctgtggtgtgcccatt**act**gagtggagtgtccaagcag-3′.

Cys962.
fP:5′-cagcatcccagctcact**tcc**ttaggttgtcttgagc-3′/rP: 5′-gctcaagacaaccctaa**gga**agtgagctgggatgctg-3′.

Cys965.
fP:5′-ctcacttgcttaggt**tct**cttgagcttattgc-3′/rP:5′-gcaataagctcaag**aga**acctaagcaagtgag-3′.

2.5. Aβ oligomer preparation

Aβ(25–35) peptide (H_2N-*Gly*^{25}-*Ser*^{26}-*Asn*^{27}-*Lys*^{28}-*Gly*^{29}-*Ala*^{30}-*Ile*^{31}-*Ile*^{32}-*Gly*^{33}-*Leu*^{34}-*Met*^{35}-COOH, the 11-mer amino acid fragment widely used in experiments as a substitute for Aβ(1–42)), and Aβ(1–42) peptide (H_2N-*Asp-Ala-Glu-Phe-Arg-His-Asp-Ser-Gly-Tyr-Glu-Val-His-His-Gln-Lys-Leu-Val-Phe-Phe-Ala-Glu-Asp-Val-Gly*^{25}-*Ser*^{26}-*Asn*^{27}-*Lys*^{28}-*Gly*^{29}-*Ala*^{30}-*Ile*^{31}-*Ile*^{32}-*Gly*^{33}-*Leu*^{34}-*Met*^{35}-*Val-Gly-Gly-Val-Val-Ile-Ala*-COOH) were dissolved in hexafluoroisopropanol to reach 1 mM concentration, dried in vacuum, and stored at −70 °C until use. Thawed Aβ(25–35) and Aβ(1–42) were diluted in PBS buffer (6.7 mM KH_2PO_4, 150 mM NaCl, pH 7.4) and subsequently incubated for 48 h at 37 °C to prepare the oligomerized Aβ followed by treatment in cultured cells as described [26]. Unless otherwise mentioned, Aβ indicates Aβ(25–35) peptide, and Aβ(42) indicates Aβ(1–42) peptide, respectively.