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Electrochemical detection of anti-tau antibodies binding to tau protein and inhibition of GSK-3β-catalyzed phosphorylation

Jose O. Esteves-Villanueva, Sanela Martic-Milne*

Department of Chemistry, Oakland University, Rochester, MI 48309, USA

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ABSTRACT

Tau protein hyperphosphorylation triggers tau aggregation and its toxicity, leading to neuronal death and cell-to-cell toxicity. Hence, inhibition of protein kinases is a viable tool toward reduction of tau toxicity. By targeting various epitopes of Tau441 protein immobilized on Au surface, the protein kinase inhibition by anti-tau antibodies was measured by surface electrochemistry. The electrochemical impedance spectroscopy was used to measure the charge transfer resistance (R_{ct}) of nonphosphorylated tau–Au film (nTau–Au) and compared with the phosphorylated tau–Au film (pTau–Au). The pTau–Au films were characterized by X-ray photoelectron spectroscopy (XPS) and time-of-flight secondary ion mass spectrometry (TOF–SIMS), which indicated high phosphorus content. The R_{ct} factor was used as the measure of inhibition efficacies by anti-tau antibodies (D8, A10, P262, and Tau46) in addition to antibody formulation intravenous immunoglobulin (IVIG). The R_{ct} factor for pTau–Au in the absence of antibodies was 0.25 ± 0.08, indicating a dramatic decrease in R_{ct} on phosphorylation. The R_{ct} factors for Tau46 and A10 were 0.57 ± 0.22 and 0.65 ± 0.26, respectively, indicating phosphorylation inhibition. All antibodies exhibited similar binding to nTau–Au. The proposed electrochemical assay may be used for detection of other posttranslational modifications.

Tau protein has a structural role in neurons, where it stabilizes microtubules, further maintaining cell integrity [1]. Normal tau is partially phosphorylated by protein kinases, but malfunctioning tau is hyperphosphorylated, which promotes detachment from microtubules and cell death [2]. Glycogen synthase kinase (GSK)-3β protein kinase is the most biologically relevant enzyme in tau phosphorylations due to its aberrant phosphorylation at multiple Ser and Thr residues. GSK-3β phosphorylation of tau has been well characterized by Western blotting and mass spectrometry [3,4]. The tau pathology has been linked to neurodegenerative diseases, including dementia and Alzheimer's disease. Currently, no cure exists for neurodegenerative diseases, but multiple avenues have been explored toward therapeutics for tauopathies. Inhibiting tau phosphorylation is one of the therapeutic avenues toward prevention of the onset of tau pathology [5]. Immunotherapies have recently gained tremendous interest due to evidence that extracellular tau causes cell-to-cell toxicity [6]. In transgenic tauopathy mice that express human tau gene mutations, immunotherapies have been implemented. In addition, the monoclonal antibodies against tau oligomers have also shown promise in reduction of tau pathology [7]. Anti-tau antibodies to the 306 to 320 sequence (R3 repeat) and the 25 to 30 sequence (N terminus) blocked uptake of tau aggregates into other cells [8,9]. The antibodies against R1 repeat 257 to 274 and R2 repeat 273 to 283 were shown to inhibit in vitro tau aggregation into paired helical filaments (PHFs), but antibodies against C-terminal 426 to 441 were ineffective [10]. Anti-tau antibodies to R2 and R3 peptide repeats reduced aggregation of full-length Tau441 and promoted clearance of preformed tau aggregates in vitro [11]. How antibodies affect tau phosphorylation has been investigated in animal models and cells. The administration of anti-tau antibodies to nonphosphorylated tau have also decreased brain levels of phosphorylated tau in mice [12]. Anti-tau antibodies to phosphorylated Ser396/Ser404 and nonphosphorylated Ser396/Ser404 regions were taken up by neurons

Abbreviations: GSK, glycogen synthase kinase; ATP, adenosine triphosphate; EIS, electrochemical impedance spectroscopy; R_{ct}, charge transfer resistance; IVIG, intravenous immunoglobulin; IgG1, immunoglobulin G1; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; Lip–NHS, lipoic acid *N*-hydroxysuccinimide ester; XPS, X-ray photoelectron spectroscopy; TOF–SIMS, time-of-flight secondary ion mass spectroscopy; nTau–Au, nonphosphorylated tau–Au; R_s, electrolyte resistance; CPE, constant phase element; W, Warburg constant; pTau–Au, phosphorylated tau–Au.

* Corresponding author.
E-mail address: martic@oakland.edu (S. Martic-Milne).

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and reduced the amount of hyperphosphorylated tau in brain slices [13]. Immunization by administering monoclonal antibodies against phosphorylated tau reduced tau phosphorylation and aggregation in animal models [14,15]. In addition, we recently reported that phosphorylation of Ser199 of tau may be reduced by using anti-tau antibodies to phosphorylated Thr231 [16]. Although anti-tau antibodies to nonphosphorylated tau had no effect on the phosphorylation of Ser199, the effects of these antibodies on other phosphosites or total phosphorylation level are unclear.

An alternative to radiolabeled detection of phosphorylation and kinase inhibitor screening is the electrochemical method due to its high sensitivity and simplicity. Electrochemical detection of tau phosphorylation has been carried out in the presence of ferrocene as a redox probe using ferrocene–adenosine triphosphate (ATP) cosubstrate [17,18]. Electrochemical impedance spectroscopy (EIS) has been used for detection of CK2-catalyzed phosphorylation of peptides [19] as well as for GSK-3β-catalyzed phosphorylation of Tau410 [20]. However, the detection of tau phosphorylation and effects of anti-tau antibodies have not been carried out before.

Here we report on the electrochemical detection of GSK-3β-catalyzed phosphorylation of Tau441 and effects of anti-tau antibodies on phosphorylation. EIS was used to determine charge transfer resistance (R_{ct}) change induced by phosphorylation and to determine the inhibitory effects by anti-tau antibodies to non-phosphorylated tau–Au film. In addition, the antigen–antibody binding was also evaluated.

1. Materials and methods

1.1. Materials

Tau441 protein (recombinant human Tau-441, 2N4R) was purchased as a lyophilized powder from rPeptide. GSK-3β was purchased from SignalChem (Richmond, British Columbia, Canada). ATP was purchased from Sigma–Aldrich (St. Louis, MO, USA).

Antibodies used in this study were D8 (mouse monoclonal anti-tau 1–150 [N-terminus sequence], Santa Cruz Biotechnology, Dallas, TX, USA), P262 (rabbit polyclonal anti-tau 259–266 [MBD repeat sequence R1], AnaSpec, Fremont, CA, USA), A10 (mouse monoclonal anti-tau 341–360 [MBD repeat sequence R4], Santa Cruz Biotechnology), and Tau46 (mouse monoclonal anti-tau 404–441 [C-terminus sequence], Abcam).

Intravenous immunoglobulin (IVIG) and immunoglobulin G1 (IgG1) were received from David Loeffler's laboratory (Beaumont Hospital, Royal Oak, MI, USA) as a kind donation.

1.2. Phosphorylation of tau by GSK-3β and ATP

Tau–Au film was incubated in 1 μl of 0.1 μg ml^{-1} GSK-3β, 18 μl of kinase buffer (40 mM Hepes [pH 7.64], 5 mM ethyleneglycoltetraacetic acid [EGTA], and 3 mM $MgCl_2$), 1 μl of 50 mM ATP, 0.5 μL of 1 x PBS, pH 7.4 for 2 h at 37 °C.

1.3. Effects of antibodies on tau phosphorylation

Tau-film was incubated in GSK-3β, kinase buffer, ATP (as described in Section 1.2.) and 0.5 μL of antibody (A10, D8, Tau46, P262, IVIG or IgG1). The final concentration of antibodies were 9 μL mL^{-1} (A10, D8, Tau46 and P262) and 45 μL mL^{-1} (IVIG or IgG1).

1.4. Electrochemical studies

The electrochemical experiments were carried out in the presence of the redox-active electrolyte: 10 mM phosphate buffer (pH 7.4), 10 mM $K_4[Fe(CN)_6]$, and 10 mM $K_3[Fe(CN)_6]$. EIS started at an open circuit potential (OCP) in the 0.01 Hz to 100 KHz frequency range for the tau–Au approach with an amplitude of 5 mV. Experimental EIS data were fit to an appropriate equivalent circuit, determining the R_{ct} using ZSimp Win 3.22 (Princeton Applied Research).

1.5. Preparation of tau–Au surface

The tau–Au surface was prepared as illustrated in Fig. 1. The clean bare Au electrodes were incubated in a 2-mM solution of lipoic acid *N*-hydroxysuccinimide ester (Lip–NHS), prepared in-house using a literature procedure, in ethanol for 1 day at 5 °C [21]. Then, the Lip–NHS–Au electrodes were incubated with 50 μg ml^{-1} tau solution (50 mM Mes [pH 8.5], 100 mM NaCl, and 0.5 mM EDTA) for 24 h at 5 °C. After that, the tau–Au modified electrodes were rinsed with a 10-mM phosphate buffer (pH 8.5). To block all unreacted NHS-active ester groups, the electrodes were then immersed in a 100-mM ethanolamine solution (in phosphate buffer, pH 7.4) for 1 h at 25 °C. The electrodes were then rinsed with 10 mM phosphate buffer solution (pH 8.5). Next, the electrodes were immersed in 10 mM 2-mercaptoethanol solution for 20 min at 25 °C to cover any free Au surface. Subsequently, the electrodes were rinsed with the 10-mM phosphate buffer solution (pH 8.5) prior to electrochemical measurements.

1.6. X-ray photoelectron spectroscopy

X-ray photoelectron spectroscopy (XPS) measurements were carried out using a Kratos Axis Ultra X-ray photoelectron spectrometer [22]. XPS can detect all elements except hydrogen and helium, probes the surface of the sample to a depth of 7–10 nm, and has a detection limit ranging from 0.1 to 0.5 at% depending on the element [23]. Survey scan analyses were carried out with an analysis area of 300 × 700 μm and a pass energy of 160 eV. High-resolution analyses were carried out with an analysis area of 300 × 700 μm and a pass energy of 20 eV. The samples were prepared using the Au sputtered silicon wafers (Nanofabrication Facility, University of Western Ontario). The silicon wafer was coated with 6 nm Ti followed by 140 nm Au. The Au wafers were cut into 1 × 1-cm substrates and cleaned by etching with piranha solution for 5 min and rinsing with deionized water. For preparation of Lip–NHS, Au substrate was immersed in 2 mM solution of Lip–NHS for 1 day at 5 °C and subsequently rinsed with ethanol. The tau–Au substrates were prepared by incubating Au substrate into 50 μg ml^{-1} tau solution (50 mM Mes [pH 8.5], 100 mM NaCl, and 0.5 mM EDTA) for 24 h at 5 °C and subsequently rinsed with buffer, to which phosphorylation agents GSK-3β, ATP, and 0.5 μl of 1× PBS (pH 7.4) solution were added. The negative control sample had no phosphorylation driving agents and was incubated with 1× PBS (pH 7.4) and kinase buffer. Phosphorylation incubation reaction was performed at 37 °C for 2 h. The data were imaged using CasaXPS software. Samples were analyzed in duplicates.

1.7. Time-of-flight secondary ion mass spectrometry

An ION–TOF time-of-flight secondary ion mass spectrometry (TOF-SIMS) IV instrument equipped with a Bi cluster liquid metal ion source was used [24]. A 25-keV Bi_3^+ cluster primary ion beam pulsed at 10 kHz was used to bombard the sample surface to generate secondary ions. The positive or negative secondary ions were extracted from the sample surface, mass separated, and detected via a reflectron type of TOF analyzer, allowing parallel detection of ion fragments having a mass/charge ratio (m/z) up to approximately 900 within each cycle (100 s). A pulsed, low-energy electron flood was used to neutralize sample charging. Negative