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Inactivation of glyceraldehyde-3-phosphate dehydrogenase by the dopamine metabolite, 3,4-dihydroxyphenylacetaldehyde

Brigitte C. Vanle, Virginia R. Florang, Daryl J. Murry, Arturo L. Aguirre, Jonathan A. Doorn*

Department of Pharmaceutical Sciences and Experimental Therapeutics, Division of Medicinal and Natural Products Chemistry, College of Pharmacy, The University of Iowa, 115 South Grand Ave, Iowa City, IA 52242-1112, United States

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ABSTRACT

Background: The aldehyde metabolite of dopamine, 3,4-dihydroxyphenylacetaldehyde (DOPAL) is an endogenous neurotoxin implicated in Parkinson's Disease. Elucidating protein targets of DOPAL is essential in understanding it's pathology. The enzyme, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a target of DOPAL.
Methods: GAPDH activity was measured via reduction of NAD^+ cofactor (340 nm). Protein aggregation was assessed with SDS-PAGE methods and specific modification via chemical probes.
Results: Low micromolar levels of DOPAL caused extensive GAPDH aggregation and irreversibly inhibited enzyme activity. The inactivation of GAPDH was dependent on both the catechol and aldehyde moieties of DOPAL. It is suggested that Cys are modified and oxidized by DOPAL.
Conclusions: The mechanism by which DOPAL modifies GAPDH can serve as a mechanistic explanation to the pathological events in Parkinson's Disease.

1. Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) catalyzes the reversible conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosphoglycerate in glycolysis [1]. GAPDH is a highly abundant and constitutively expressed enzyme and is utilized as an internal standard for quantitative protein comparison. However, recent work has shown that deficiencies in GAPDH activity or expression are possible factors in neurodegenerative disorders such as Alzheimer's and Parkinson's Disease (PD) [2]. In the PD rotenone mouse model, GAPDH activity was overexpressed and activity was potently inhibited [3]. Several neurodegenerative diseases are characterized by the accumulation of protein aggregates [4]. In particular, PD involves the selective loss of dopaminergic neurons in the substantia nigra and formation of proteinaceous Lewy bodies [5]. Accumulating evidence suggests that GAPDH is central to the formation of abnormal toxic protein aggregates or Lewy bodies in PD pathology. GAPDH promoted formation of Lewy bodies and co-localized with alpha-synuclein [6] and n *ex vivo* study showed that Lewy bodies in post-mortem PD brains were immunoreactive for GAPDH [7]. Evidence has demonstrated that GAPDH may even promote *in vivo* formation of these toxic protein aggregates [8]. The neurons affected in PD produce dopamine (DA), a neurotransmitter involved in vital physiological functions, including coordination of voluntary movement mood regulation [9].

The vulnerability of dopaminergic neurons in PD is currently unknown. Elucidating mechanistic links and protein targets are of high interest as potential biomarkers of PD pathogenesis. To account for the selective neurodegeneration observed in PD, it is hypothesized that a toxin endogenous to DA neurons is involved in the degeneration of these neurons [10,11].

The major route of DA metabolism is deamination by monoamine oxidase (MAO), to form hydrogen peroxide and 3,4-dihydroxyphenylacetaldehyde (DOPAL). Metabolism of DOPAL is by aldehyde dehydrogenase (ALDH2) to 3,4-dihydroxyphenylacetic acid (DOPAC) and by cytosolic aldehyde reductase (AR) to 3,4-dihydroxyphenyletanol (DOPET) [10]. Due to its high

Abbreviations: ALDH2, aldehyde dehydrogenase 2; AR, aldehyde or aldose reductase; AS, alpha-synuclein; DA, dopamine; DAQ, dopamine quinone; DHPAN, 3,4-dihydroxyphenylacetonitrile; DOPAC, 3,4-dihydroxyphenylacetic acid; DOPAL, 3,4-dihydroxyphenylacetaldehyde; DOPET, 3,4-dihydroxyphenylethanol; DTNB, 5,5′-dithiobis-(2-nitrobenzoic acid); G3P, glyceraldehye-3-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSH, glutathione; MAO, monoamine oxidase; MOPAL, 3-methoxyphenylacetaldehyde; NAL, n-acetyl lysine; NBT, nitro-blue tetrazolium.

* Corresponding author.
E-mail address: jonathan-doorn@uiowa.edu (J.A. Doorn).

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electrophilicity, DOPAL modifies proteins [12–14] and is toxic to neurons [12,15–17]. In fact, DOPAL is several of magnitudes more toxic than DA *in vitro* and *in vivo* [10,18]. Toxicity of DOPAL is also significantly increased by auto-oxidation of the catechol to the quinone and subsequent formation of radical species [19]. DOPAL auto-oxidation greatly enhances protein cross-linking and produces superoxide radicals. Due to the presence of redox-sensitive thiols on GAPDH, it is utilized as a model for cysteine modification by electrophiles. It was extensively modified by dopamine-quinone (DAQ) [20], 4-hydroxy-2-nonenal [21]. In light of the structural similarity between DA, DAQ and DOPAL, it was hypothesized that DOPAL would similarly modify and inactivate GAPDH. We have previously used GAPDH as a protein model for cross-linking with DOPAL [14,19]. The goals of this work are to elucidate the interaction between DOPAL and GAPDH, measure enzyme activity, mode of binding, and determine the specific sites of modification by DOPAL.

2. Materials and methods

2.1. Chemicals

DOPAL was biosynthesized via rat liver MAO procedure as previously established [15]. Stock concentrations were determined using an ALDH assay with nicotinamide adenine dinucleotide (NAD^+) and HPLC [16]. MOPAL was biosynthesized as previously outlined [14]. The following compounds: DA, DOPAC, GAPDH from rabbit muscle, G3P, acetonitrile (ACN), glutathione (GSH) and all other chemicals were acquired from Sigma-Aldrich (St. Louis, MO), unless otherwise noted. The DOPAL analogs, 3,4-dihydroxyphenylacetonitrile (DHPAN) and 3-methoxy-4-hydroxyphenylacetaldehyde (MOPAL) were synthesized as previously described [12,22], respectively.

2.2. Protein analysis by SDS-PAGE

Rabbit muscle GAPDH (0.3 mg/mL) was incubated with DOPAL (50 mM sodium phosphate buffer, pH 7.4, 37 °C) at the given time periods. Protein samples were denatured by addition of 6× gel loading buffer (11 mM EDTA, 3.3 mM Tris-HCl, 0.017% SDS, 0.015% bromophenol blue) and heated to 80 °C for 3 min. Briefly, 9 μg of protein was loaded and 10% polyacrylamide gel was used to separate protein bands via SDS-PAGE. Protein bands were stained with Coomassie blue.

2.3. Analysis of catechol adducts on protein

Protein samples were stained with the redox-cycling sensitive dye nitroblue tetrazolium (NBT). GAPDH and DOPAL or MOPAL or DHPAN were incubated as stated before for 4 h. GAPDH samples (5 μg) were subjected to 10% acrylamide SDS-PAGE and transferred to nitrocellulose membrane as previously described [14,23,24]. The membrane was placed in 0.24 mM NBT with 2 M potassium glycine buffer (pH 10) and incubated overnight at 4 °C.

2.4. GAPDH activity assay

GAPDH enzymatic activity was measured by NADH production at 340 nm for 3 min [25]. After incubation of GAPDH (0.3 mg/mL) with and without DOPAL or DOPAL analogs, 100-μL aliquots were transferred to a 96-well plate with a final volume of 200-μL, containing: 50 mM sodium phosphate buffer at either pH 7.4 or 8.0, 1 mM NAD^+, and 1 mM EDTA. The reaction was initiated by adding 0.8 mM G3P to each well. Enzyme activity in the presence of DOPAL was expressed as the percentage of GAPDH activity without DOPAL.

2.5. Enzyme activity recovery assay

GAPDH was incubated with DOPAL as previously described in *activity assay*. Following incubation, unbound DOPAL was removed by filtration with size–exclusion spin columns (Micro Bio-Spin) according to manual (Bio-Rad laboratories) and spun for 3 min at 3000 × g. Aliquots (100-μl) were transferred from mixtures with and without filtration, and activity was measured as described in *activity assay*. Activity at each DOPAL concentration was measured with and without filtration to control for binding of enzyme to column resin. To measure inhibition recovery as a product of GAPDH thiols, GSH (0.5 mM) was briefly added to each incubation mixture prior to activity measurement.

2.6. Western blot analysis

GAPDH (1 μg) was loaded in 10% polyacrylamide SDS-PAGE. After electrophoresis, protein was transferred to nitrocellulose membrane at 20 V for 45 min and blocked overnight in 5% BSA + TBST. Primary rabbit *anti*-GAPDH antibody was diluted to 1:10,000 (Sigma Aldrich) for 2 h at room temperature. The nitrocellulose membranes were washed with 0.05 M Tris, 0.9% NaCl containing 0.05% Tween-20 (TBS-T). A horseradish-peroxidase-conjugated secondary goat-*anti*-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was diluted to 1:20,000 and incubated for 1.5 h at room temperature. Protein bands were detected with an Amersham ECL-plus Western Blotting Detection kit, according to manufacturing instructions.

2.7. Analysis of free thiols

GAPDH (0.5 mg/mL) was incubated with 25 or 125 μM DOPAL in 50 mM sodium phosphate buffer pH 7.4 for 2 or 4 h. After incubation, samples were titrated with 1 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) for 20 min at room temperature, and heated for 3 min at 80 °C, samples were then centrifuged at 1000 g for 3 min and 100-μL of supernatant was transferred to a 96-well plate. Absorbance was measured for the reduced 2-nitro-5-benzoic acid product at 412 nm, which directly correlates amount of free thiols in solution.

2.8. LC-MS identification of oxidized peptides

GAPDH (0.3 mg/mL) and GAPDH-DOPAL (0.3 mg/mL and 50 μM) were incubated in glass vials for 4 h at 37 °C. Excess DOPAL was removed by filtration columns. Final concentrations of $NaCNBH_3$ (1 mM) and ACN (0.1%) v/v were added, samples were briefly heated for 3 min at 70° C, and trypsin digested (Promega) in solution for 8 h at 37 °C at a 1:50 ratio. A 10-μL aliquot was taken and diluted 1:10 with 50 mM ammonium carbonate and 15 μL of sample was injected onto a Phenomenex Aeris Widepore XB-C18 column (150 × 2.1 mm, 3.6 μm). Mobile phase A was 0.1% formic acid in water, mobile phase B was 0.1% formic acid in ACN. The gradient elution was 0 min: 5% B, 5–15 min: 5–50% B, 15–30 min: 50–90% B, 30–35 min: 90%, 36 min: 50%, 37 min: 5%. A column oven (CTO-20A, Shimadzu Scientific) was set to 40 °C. Total flow was set to 0.2 mL/min. The MS instrument was a Shimadzu LCMS–IT-TOF (Shimadzu Scientific Instruments, Columbia, MD) using electrospray ionization (ESI) in positive ion detection mode. The ESI interface temperature and heat block were set at 200 °C and the nebulizing gas flow at 1.5 L/min with makeup gas on. The instrument scanned from 200 to 2000 *m/z* with a 0.3 s loop time. MS spectra were analyzed by comparison to UCSF Prospector *Digest* command which matched the experimental *m/z* masses to theoretical sequenced GAPDH peptides. The database was set to assume

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