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Original research article

Gambogic amide selectively upregulates TrkA expression and triggers its activation

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ABSTRACT

Background: Gambogic amide is the first identified small molecular agonist for TrkA receptor. It mimics NGF functions by selectively activating TrkA receptor and preventing neuron death. However, its function different from that of NGF remains unknown.
Methods: In the current study, we detect the effect of gambogic amide on TrkA expression using TrkA-expressing cell lines *in vitro* and hippocampi from mice treated with gambogic amide.
Results: We have confirmed that gambogic amide displays robust neurotrophic activities in provoking neurite outgrowth *in vitro*. However, gambiogic amide displays a different kinetics from NGF in activating TrkA signals. NGF swiftly provokes TrkA activation and quickly induces TrkA degradation, while gambogic amid selectively upregulates TrkA protein and mRNA levels in a time-dependent manner. Administration of this compound in mice also activates TrkA receptor in hippocampus and promotes TrkA transcription and expression.
Conclusion: This study provides a novel mechanism of how gambogic amide regulates TrkA receptor, other than mimicking NGF in triggering TrkA activation.

Introduction

Nerve growth factor (NGF), the prototype of neurotrophin family members, binds to the cognate receptor, TrkA, and triggers the activation of numerous signaling cascades and promotes the survival and differentiation of sensory and sympathetic neurons [1]. It is known that the loss of TrkA density correlates with neuronal atrophy and severe cognitive impairment such as that associated with Alzheimer's disease (AD) [2]. Both *in vitro* and animal model studies demonstrate that NGF might be clinically valuable for treatment of peripheral or CNS diseases [3–5]. However, the clinical application of NGF as a therapeutic agent is limited because of its difficulties in crossing the blood–brain barrier, poor pharmacokinetic properties, and other problems associated with systemic treatment [3,6].

Gambogic amide has been reported to mimic NGF's prosurvival functions, prevent glutamate-induced neuronal cell death and provoke prominent neurite outgrowth in PC12 cells. Administration of this molecule in mice substantially diminishes kainic acid-triggered neuronal cell death and decreases infarct volume in the transient middle cerebral artery occlusion model of stroke [7]. Thus, gambogic amide might provide effective treatments for neurodegenerative diseases and stroke. The activation of TrkA receptor seems to be triggered by direct interaction since gambogic amide has been reported to selectively bind to the juxtamembrane domain of TrkA [7]. In this study, we have confirmed previous reports that gambogic amide displays robust neurotrophic activities in provoking neurite outgrowth *in vitro*. However, interestingly, we have found that unlike NGF, gambogic amide selectively upregulates TrkA transcription and translation, in addition to mimicking NGF to trigger TrkA receptor activation. Moreover, gambiogic amide displays a different kinetics from NGF in activating TrkA signals. NGF swiftly induces TrkA activation and the signal quickly fades away, associated with prominent degradation of TrkA receptor, while treatment with gambogic amide, but not its inactive analog gambogic acid, selectively

Abbreviations: AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; DRG, dorsal root ganglia; GA, gambogic acid; FBS, fetal bovine serum; NGF, nerve growth factor; NT, neurotrophin; Trk, tropomyosin-related kinase.

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upregulates TrkA protein levels *in vitro* by escalating its transcription, and induces robust and sustained activation of the downstream signals. Administration of this molecule in mice also activates TrkA receptor in hippocampus, and selectively upregulates its expression at both the mRNA and protein levels. Thus, in addition to mimicking NGF to activate TrkA, gambogic amide exerts potent neurotrophic activities by upregulating TrkA protein as well.

Materials and methods

Animal ethics

Animal experiments were conducted according to the institutional ethical guidelines for animal experiments and approved by the Institutional Animal Care and Use Committee (IACUC) at Emory University.

Cell culture

Rat pheochromocytoma 12 cells were cultured in RPMI-1640 medium, supplemented with 10% horse serum and 5% fetal bovine serum (FBS). P23A cells were derived from SY5Y cells and endogenously express TrkA receptors. Both P23A cells and human chronic myelogenous leukemia K562 cells were cultured in RPMI-1640 medium, supplemented with 10% FBS. All culture media were supplemented with 2 mM L-glutamine, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin.

Primary neurons and dorsal root ganglion culture

Primary rat cortical neurons were cultured as previously described [8]. Ten days plated neurons were exposed to 25 nM of gambogic amide (Spectrum Collection, MicroSource Discovery System, Gaylordsville, CT, USA) or gambogic acid (Spectrum Collection) for indicated time. Dorsal root ganglia were harvested according to the previous report with minor modification [9]. Briefly, DRGs were gently dissected from embryonic day 18 (E18) timed pregnant Sprague-Dawley rats and incubated in a solution of collagenase II (200 U/mL, Roche Diagnostics, Indianapolis, IN, USA) and dispase II (2.5 U/mL, Roche Diagnostics) in HBSS. Cells were centrifuged at a low speed, washed and gently triturated in HBSS-CMF three times. The dissociated DRGs were then cultured in DMEM medium with 10% FBS and penicillin/streptomycin in a 24-well plate coated with poly-D-lysine (sigma) and mouse laminin (Invitrogen). DRG explants were treated with 50 ng/ml of NGF or 25 nM of gambogic amide for 48 h.

Gambogic amide administration

Male C57BL/6 mice aged of two months were intraperitoneally injected with 0.5 mg/kg or 4 mg/kg of gambogic amide, once daily for 5 days. Gambogic amide was dissolved in 20% ethanol/N.S. The control mice were injected with the vehicle solution. Twenty-four hours after the last administration, the mice were sacrificed and the hippocampi were isolated and grouped ($N = 5$). The hippocampal tissue was homogenized in RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1 mM NaF; 1 mM sodium orthovanadate, 1% NP-40) in the presence of 1× phosphatase inhibitor cocktail, protease inhibitor cocktail (Sigma Aldrich), and centrifuged at 12,000 r.p.m. for 15 min. The supernatant (50 μg) was employed for SDS-PAGE and immunoblotting analysis with indicated antibodies, respectively. For semi-quantitative RT-PCR analysis, partial hippocampi were treated with TRIZOL reagent (Invitrogen) and total RNA was extracted according to the manufacture's manual.

Neurite outgrowth assay

In a typical neurite outgrowth experiment, PC12 cells or P23A cells were seeded in the growth medium at 5×10^4 cells per well in poly (L-lysine)-coated 12-well culture plates (BD Biosciences, San Jose, CA, USA), allowed to grow for 24 h, and supplemented with indicated concentration of gambogic amide or NGF. Two days later, neurite outgrowth was measured according to the distance between the cell periphery and the tip of neurite, and neuritic processes longer than two cell bodies in length were calculated as neurites. At least 30 cells were measured and results were represented as the three independent experiments. Statistical significance was determined with independent *t*-test. The data were presented as mean ± S.D.

Western blot analysis

Cells or tissue homogenates were lysed with RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 1 mM NaF; 1 mM sodium orthovanadate, 1% NP-40) in the presence of phosphatase inhibitor cocktail, protease inhibitor cocktail (Sigma Aldrich) and centrifuged at 12,000 r.p.m. for 15 min. Protein concentration was determined by using a Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Twenty micrograms (for cells) or 50 μg (for tissues) of proteins were loaded onto SDS-PAGE and blotted onto nitrocellulose filters (GE Healthcare, Fairfield, CT, USA). Membranes were stained with antibodies anti-TrkA, anti-pTrkA (Y785), anti-EGFR, anti-P75NTR and anti-NGF (Cell Signaling Technology, Danvers, MA, USA), anti-Trk (C14) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TrkB (BioVision) or anti-α tubulin antibody (Sigma Aldrich). All the antibodies were diluted according to the data sheet. HRP-conjugated anti-rabbit IgG (GE Healthcare) or HRP-conjugated anti mouse IgG (Santa Cruz Biotechnology) were used as secondary antibodies at a final dilution of 1: 10,000. The reactions were visualized by the ECL detection system as recommended by the manufacturer (GE Healthcare).

Semi-quantitative RT-PCR analysis

Total RNA was extracted from cells or tissues with TRIZOL reagent (Invitrogen), according to the manufacturer's instructions. Reverse transcription PCR experiments were carried out with cDNAs generated from 2 μg of total RNA using a GeneAmp RNA PCR kit (Applied Biosystems, Foster City, CA, USA). The RT-PCR exponential phase was determined on 25 cycles to allow semi-quantitative comparisons of cDNAs developed from identical reactions with TaKaRa ExTaq polymerase (TaKaRa Bioscience, Kyoto, Japan). Following are the primers used:

TrkA: 5′-ctgtcaaggcactgaaggag-3′; 5′-aggtgaagatctcccagag-3′
TrkB: 5′-atgtcgccctggccgaggtgg-3′; 5′-gcaatacaaatcctgagtgt-3′
P75NTR: 5′-cctgtcctgtcccacattcc-3′; 5′-cttcacaaatgccctgtggc-3′
Mouse GAPDH: 5′-accacagtccatgccatcac-3′; 5′-caccaccctgttg ctgtagcc-3′

Results

Gambogic amide mimics NGF in provoking neurite outgrowth of dorsal root ganglia, PC12 cells or P23A cells cultured in vitro.

Previous studies have demonstrated that gambogic amide mimics NGF's functions in provoking neurite outgrowth of PC12 cells when incubated with 0.5 μM compound for 5 days.