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Differential regulation of human tyrosine hydroxylase isoforms 1 and 2 in situ: Isoform 2 is not phosphorylated at Ser35

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ABSTRACT

The major human tyrosine hydroxylase isoforms (hTH1 and 2) differ in their ability to be phosphorylated in vitro. hTH1 is phosphorylated at Ser31 by extracellular signal-regulated kinase (ERK). This kinase is not capable of phosphorylating hTH2 at Ser35 (the residue that corresponds to Ser31 in hTH1). We have stably transfected SH-SY5Y cells with hTH1 or hTH2 to determine if hTH2 can be phosphorylated at Ser35 in situ. Forskolin increased the phosphorylation of Ser40 in hTH1 and Ser44 in hTH2. Muscarine increased the phosphorylation of both Ser19 and Ser40/44 in both hTH1 and hTH2. EGF increased the phosphorylation of Ser31 in hTH1. Phosphorylation of Ser35 in hTH2 was not detected under any of the conditions tested. Inhibition of ERK by UO126 decreased the phosphorylation of Ser31 and this lead to a 50% decrease in the basal level of phosphorylation of Ser40 in hTH1. The basal level of Ser44 phosphorylation in hTH2 was not altered by treatment with UO126. Therefore, phosphorylation of Ser31 contributes to the phosphorylation of Ser40 in hTH1 in situ; however, this effect is absent in hTH2. This represents a major difference between the two human TH isoforms, and has implications for the regulation of catecholamine synthesis in vivo.

1. Introduction

Tyrosine hydroxylase (TH) [EC1.14.16.2], the rate-limiting enzyme in catecholamine biosynthesis [1], is subject to a wide variety of regulatory mechanisms [2]. Long-term regulation of TH activity is primarily controlled by modulation of TH protein levels [2]. Acute regulation of TH activity occurs via two distinct forms of feedback inhibition by the catecholamines [3] and also by phosphorylation of three key serine (Ser) residues: Ser19, Ser31 and Ser40 [4].

The binding of catecholamines to TH results in a 78–98% inhibition of enzyme activity in vitro [3]. Phosphorylation of Ser40 directly increases TH activity by inducing a 500-fold increase in the rate of dissociation of the catecholamines from the high affinity site of TH [5], thereby relieving catecholamine inhibition of the enzyme [6]. Stimulation of Ser40 phosphorylation results in an increase in TH activity and catecholamine synthesis in situ and in vivo [4]. While the role of phosphorylation of Ser40 is well-understood, the function of phosphorylation of Ser19 and Ser31 has only recently been elucidated.

Phosphorylation of Ser19 does not directly increase TH activity in vitro [7]; however, incubation of Ser19-phosphorylated TH with 14-3-3 protein is able to produce a small increase in enzyme activity [8,9]. Phosphorylation of Ser31 induces a small (1.2–2-fold) increase in TH activity in vitro, primarily by decreasing the K_M for the cosubstrate tetrahydrobiopterin (BH_4) [10,11]. In addition to these small effects on TH activity, prior phosphorylation of Ser19 or Ser31 is able to increase the rate of phosphorylation of Ser40 by approximately 3-fold and 9-fold, respectively, in vitro in a process known as hierarchical phosphorylation [12,13]. Phosphorylation of Ser19 or Ser31 potentiates both the phosphorylation of Ser40 and the pSer40-induced increase in TH activity in situ [13,14].

TH is encoded by a single gene. While most species express only a single isoform of TH [15], there are 4 human TH (hTH) isoforms (hTH1-4) which differ only in the number of amino acids N-terminal to the Ser31 residue in hTH1 [16,17]. The sequence surrounding the other phosphorylation sites is identical in all TH isoforms. hTH1 is the smallest of the hTH enzymes, and is homologous to TH found in other species [18]. hTH2 contains an additional 4 amino acids inserted immediately N-terminal to Ser31, hTH3 contains an additional 24 amino acids, while hTH4 contains both inserts (4+27). All four isoforms are expressed in human brain and adrenals [19,20], with hTH1 and hTH2 being the two major isoforms, together comprising approximately 90% of TH in brain [20,21].

hTH1 is phosphorylated at Ser31 in vitro by extracellular signal-regulated protein kinase (ERK) [11,13]; however, hTH2 cannot be phosphorylated at the equivalent Ser31 residue (Ser35) by ERK [11,13]. The addition of four amino acids N-terminal to this residue in hTH2 was believed to change the kinase specificity of the site from an ERK site to a calcium/calmodulin-dependent protein kinase II (CaMKII) site [22]. However, in other studies CaMKII was unable to

Abbreviations: TH, tyrosine hydroxylase; Ser, serine; hTH, human TH; BH_4, tetrahydrobiopterin; ERK, extracellular signal-regulated protein kinase; CaMKII, calcium/calmodulin-dependent protein kinase II; DMEM, Dulbecco's Modified Eagle Medium

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phosphorylate the Ser35 residue in hTH2 in vitro [13]. It is unknown whether hTH2 can be phosphorylated at Ser35 in intact systems.

Due to the important role that phosphorylation of Ser31 plays in regulating the rate of Ser40 phosphorylation, the absence of phosphorylation of Ser35 in hTH2 represents a major difference between the two isoforms [13]. We have stably transfected the neuroblastoma cell line SH-SY5Y with hTH1 and hTH2 isoforms and stimulated the cells in an effort to determine whether hTH2 is able to be phosphorylated at Ser35 in situ and investigate the influence that this may have on differential regulation of the two isoforms in situ.

2. Materials and methods

2.1. Materials

pcDNA3.1, Lipofectamine 2000 and geneticin were from Invitrogen (Carlsbad, CA, USA). Wizard SV gel and PCR clean-up system and UO126 were from Promega (Madison, WI, USA). XL1-blue competent cells were from Stratagene (La Jolla, CA, USA). Quicklyse plasmid purification mini-prep kit and plasmid purification maxi-prep kit were from Qiagen (Hilden, Germany). SH-SY5Y cells were obtained from ATCC (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) was from GIBCO (Carlsbad, CA, USA). Fetal calf serum was from Bovogen (Essendon, Australia). Rat-tail collagen, forskolin, muscarine chloride and mouse anti-β-actin antibody were from Sigma-Aldrich (St Louise, MO, USA). Epidermal growth factor was from Affinity BioReagents (Golden, CO, USA). L-[3,5-^{3}H]-tyrosine was from GE Healthcare (Buckinghamshire, England). Peptides were synthesized by Auspep (Parkville, Australia). Sulfolink immobilization kit and Aminolink plus immobilization kit were from Pierce (Rockford, IL, USA).

2.2. Preparation of pcDNA3.1 hTH1 and hTH2

pcDNA3.1 hTH1 was generously supplied by Ingo Lehmann. For pcDNA3.1 hTH2, hTH2 cDNA was amplified from pET3a hTH2 by PCR using primers constructed to encompass the *Bam*HI and *Eco*RI sites (forward primer = CGTAGGATCCATGCCCACCCCCGACGCAACC, reverse primer = CGTAGAATTCCTAGCCAATGGCACTCAGCGC). The PCR product was purified using the Wizard SV gel and PCR clean-up system according to the manufacturer's instructions. The purified PCR product and pcDNA3.1 were digested with *Bam*HI and *Eco*RI, and then purified using the Wizard SV gel and PCR clean-up system. The purified hTH2 and pcDNA3.1 digests were then ligated together, and transformed into XL1-Blue competent cells. Transformed bacteria were selected using LB agar plates containing 100 μg/mL ampicillin. Overnight cultures were inoculated from single colonies, and plasmids purified using the Quicklyse plasmid mini-prep kit according to the manufacturer's instructions. Insertion of hTH2 cDNA into pcDNA3.1 was confirmed by DNA sequencing.

Stocks of pcDNA hTH1 and pcDNA hTH2 were prepared using the plasmid purification maxi-prep kit according to the manufacturer's instructions, and were stored at −20 °C in DNAse/RNAse-free H_2O.

2.3. Cell culture

Transfected and wild-type SH-SY5Y cells were routinely maintained in 10% DMEM (DMEM supplemented with 10% fetal calf serum, 10 mM Hepes and 2 mM L-Glutamine) at 37 °C, 5% CO_2 in a humidified incubator. Cells were not allowed to exceed 90% confluency before passaging, and were not used above passage 20.

6-well and 12-well plates were coated with 10 μg/mL rat-tail collagen in phosphate-buffered saline for 2 h at 37 °C, and then washed with phosphate-buffered saline, prior to plating of cells. Cells were plated in collagen-coated plates (unless otherwise stated) at a density of 4–7 × 10^5 cells/well for 6-well plates, and 2–4 × 10^5 cells/well for 12-well plates, and were maintained in 10% DMEM until 80% confluent.

2.4. Stable transfection of SH-SY5Y cells

SH-SY5Y cells were plated in 12-well (non collagen-coated) plates at a density of 4 × 10^5 cells/well, and were incubated with 1.6 μg pcDNA3.1 hTH1 or pcDNA3.1 hTH2 and 4 μL Lipofectamine 2000 reagent for 24 h, before media were aspirated and replaced with 10% DMEM supplemented with 0.06 mg/mL geneticin (DMEM-G). Cells were maintained in DMEM-G; during this time, cell density was observed to decrease to <1% confluency, and then increase until 80% confluent. In wild-type (non-transfected) SH-SY5Y cells, incubation in DMEM-G resulted in 100% cell death. Cells were transferred to a 5 mL flask, and were maintained in DMEM-G until cells were transferred to a 75 mL flask, after which point hTH1 and hTH2 SH-SY5Y cells were routinely maintained in 10% DMEM. TH protein expression was analyzed using western blotting as described below.

2.5. TH activity assay

Cells were plated in 6-well plates. Cells were washed in serum-free media (DMEM supplemented with 10 mM Hepes and 2 mM L-Glutamine), and then pre-incubated in serum-free media at 37 °C, 5% CO_2 for 2 h. Media were aspirated, cells were washed in phosphate-buffered saline and 350 μL of homogenization buffer (50 mM Tris, pH 7.5, 1 mM EGTA, 1 mM EDTA, 1 mM dithiothreitol, 80 μM ammonium molybdate, 1 × protease inhibitor cocktail, 1 mM tetrasodium pyrophosphate, 5 mM β-glycerophosphate, 1 mM sodium vanadate and 1 μM microcystin) was added to each well. Wells were scraped, and cells were lysed by sonication. Samples were centrifuged at 18,000×*g* for 15 min at 4 °C, and the supernatants were collected and assayed for TH activity using a variation of the tritiated-water release assay [23] as described [14]. Briefly, TH activity assays were initiated by the addition of an equal volume of reaction mix (60 mM potassium phosphate buffer, pH 7.4, 0.006% (v/v) 2-mercaptoethanol, 36 μg/mL catalase, 24 μM tyrosine, 4 μCi L-[3,5-^{3}H]-tyrosine/mL and 20 μM BH_4, final concentrations). Background samples did not contain BH_4. Assays were performed at 30 °C for 9 min. Reactions were linear under these conditions. To determine TH protein levels, SDS-PAGE and western blotting were performed on cell extracts alongside a dilution series of recombinant TH calibration standards as described below.

2.6. Treatment of SH-SY5Y cells

hTH1 SH-SY5Y and hTH2 SH-SY5Y cells were plated in 6- or 12-well plates. Cells were pre-incubated for 2 h in serum-free media prior to cell treatment as described above. Cells were treated with 10 mM forskolin, 10 mM muscarine, 50 ng/mL EGF or relative vehicle for control wells for 5 or 30 min. The vehicle for muscarine and EGF treatments was H_2O, and the vehicle for forskolin was DMSO. Forskolin and DMSO were added to cells such that the final concentration of DMSO did not exceed 0.1%. For pre-incubation with UO126, cells were pre-incubated in serum-free DMEM for 1.5 h and then were incubated with 10 μM UO126 for 30 min.

2.7. Preparation of phospho-specific antibodies and total TH antibody

To generate antibodies to detect TH phosphorylated at Ser40, Ser31 or Ser19, we synthesized phospho-peptides corresponding to residues 36–44 (GRRQpSLIED; pSer40TH), 27–35 (EAVTpSPRFI; pSer31TH) and 15–23 (RRAVpSEQDA; pSer19TH) of rat TH with the addition of a terminal cysteine residue as described [24]. After purification by HPLC, an aliquot of each phospho-peptide was linked to diphtheria toxoid and used to immunize rabbits (for pSer19 and