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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

pSer31) and sheep (for pSer40). After the animals had been bled, serum was collected and antibodies were purified via phosphopeptide affinity chromatography. Two milligrams of each phosphopeptide was coupled to 2 mL of Sulfolink coupling gel using the Sulfolink immobilization kit for peptides. Sera were purified according to the manufacturer's general protocol for affinity purification of protein (Pierce). The specificity of the phospho-specific antibodies was assessed by immunoblotting using recombinant rat TH specifically phosphorylated to full stoichiometry at Ser40 (by protein kinase A), Ser31 (by ERK1), Ser19 (mutated form of rat TH (Ser40Ala) phosphorylated by CaMKII) and also non-phosphorylated recombinant rat TH as described [13]. As shown in Fig. 1A, the phospho-specific antibodies recognized their respective phospho-rTH proteins in a concentration-dependent manner. The phospho-specific antibodies did not significantly cross-react with non-phosphorylated rTH, or with rTH phosphorylated at the alternative Ser residues. hTH1, hTH2 and hTH3/4 have different amino acid sequences immediately N-terminal to their relative Ser31 residues. The pSer31 antibody was therefore further assessed for the detection of the different human TH isoforms phosphorylated at the equivalent Ser31 residues (Fig. 1B). The pSer31 antibody recognized recombinant hTH1, hTH3 and hTH4 phosphorylated at the equivalent Ser31 residues by ERK; hTH2 cannot be phosphorylated by ERK in vitro and thus was not analyzed [11, 13]. To determine if the pSer31 antibody recognizes the pSer35 hTH2 sequence, the pSer31 antibody was incubated for 1 h at 25 °C with 500 nM of pSer35 hTH2 peptide (VRGQpSPRFI), 500 nM pSer31TH peptide or 500 nM pSer40TH peptide. Both the pSer35 hTH2 peptide and the pSer31TH peptide were able to block the binding of the pSer31 antibody to the pSer31 TH immunoblot, while the pSer40TH peptide did not block binding (Fig. 1C). To further examine the relative binding of the pSer31 antibody to the pSer35 hTH2 and pSer31TH peptides, the antibody was incubated with different concentrations of the two peptides. Both peptides were able to block the binding of the antibody to the pSer31 TH immunoblot to a similar extent (Fig. 1D). These data demonstrate that the pSer31 antibody is able to recognize hTH1, 3 and 4 phosphorylated at their equivalent Ser31 residues despite their different N-terminal amino acid sequences, and if hTH2 was phosphorylated at Ser35, then the pSer31 antibody could recognize it.

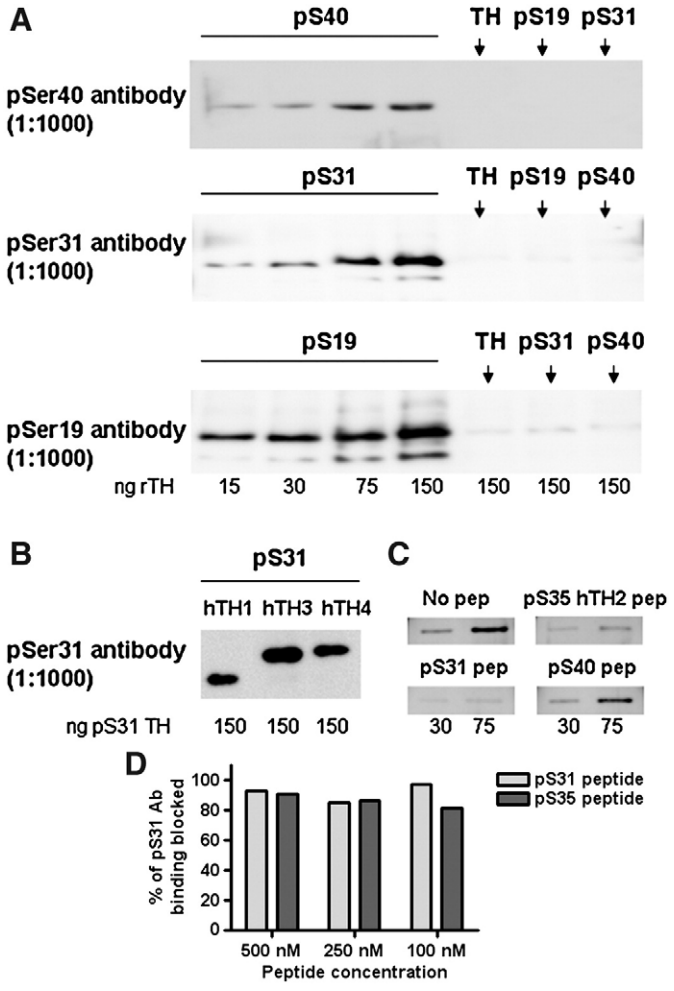

Fig. 1. Characterization of phospho-specific antibodies. (A) The specificity of phospho-specific antibodies was assessed by immunoblotting using different loadings of recombinant rat TH that was either non-phosphorylated (TH), or phosphorylated specifically at Ser40 (pS40), Ser31 (pS31) or Ser19 (Ser40Ala rTH phosphorylated by CaMKII, pS19). (B) The ability of pSer31 antibody to detect human TH (hTH) isoforms 1, 3 and 4 phosphorylated by ERK (pS31 TH, 150 ng loaded) was assessed by immunoblotting. (C) pSer31 antibody was incubated without peptide (no pep), or with pSer35 hTH2, pSer31 TH or pSer40 TH peptides. The binding of the blocked antibody to pS31 TH (30 and 75 ng loaded) was assessed by immunoblotting. (D) pSer31 antibody was incubated without peptide, or with 100, 250 or 500 nM of pSer31 TH or pSer35 hTH2 peptides. The binding of the blocked antibody to pS31 TH was assessed by comparing the densitometric signal from the blocked antibody to that of the non-blocked antibody. Results are expressed as a percentage of pSer31 antibody (pS31 Ab) binding blocked by the relative peptides.

To generate antibodies to detect non-phosphorylated TH, rabbits were immunized with recombinant human TH protein lacking the first 156 residues to avoid any interaction of the antibody with the N-terminal regulatory domain, where phosphorylation could alter the binding to the antibody to the TH protein. Animals were bled and serum was collected. Two milligrams of recombinant rat TH was coupled to Aminolink coupling gel using Aminolink plus immobilization kit. Antibodies were purified according to the manufacturer's general protocol for affinity purification of protein (Pierce). The total TH antibody recognized recombinant hTH1, hTH2, hTH3 and hTH4 in a concentration-dependent manner, and recognized each of the 4 isoforms to an equal extent (data not shown).

2.8. SDS-PAGE and western blotting

Following incubation of cells with various stimuli, media were aspirated and treatments were terminated by the addition of Laemlli sample buffer (50 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 1% DTT, 2 mM EDTA). Samples were applied to 10% SDS-polyacrylamide gels before being transferred to nitrocellulose as described [25]. To determine the stoichiometry of Ser phosphorylation and TH protein levels, recombinant hTH phosphorylated to full stoichiometry at Ser19, Ser31 and Ser40 (as described above) was used as a calibration standard. hTH1 and hTH2 SH-SY5Y cell extracts were run along with a dilution series of the calibration standards. Nitrocellulose membranes were then immunoblotted using phospho-specific or total TH antibodies as described previously [24], or with anti-β-actin to provide a protein loading control. Analysis of site-specific TH phosphorylation was performed as previously described [14]. Immunoblots were visualized using the Image Reader LAS-3000 (Fujifilm) imaging system using ECL plus detection reagents. The density of total TH or phospho-specific TH bands was measured using MultiGauge V3.0 (Fujifilm).

2.9. Statistical analyses

The data were expressed as a fold-increase of the mean of the control samples (control = 1), and are presented as mean ± SEM for the stated number of experiments. Statistical significance was assessed using one-way ANOVA followed by Tukey's test for multiple comparisons or Student's unpaired *t*-tests for analysis of Ser31 phosphorylation and basal phosphorylation stoichiometry.

3. Results

3.1. Stable transfection of hTH1 and hTH2 into SH-SY5Y cells

SH-SY5Y cells were stably transfected with hTH1 or hTH2 cDNA inserted in the pcDNA3.1 mammalian expression vector. The expression of hTH1 and hTH2 was confirmed by SDS-PAGE and western blotting, and also by the measurement of TH activity. TH protein was not observed in wild-type SH-SY5Y cells (Fig. 2A), and these cells contained no detectable TH activity (Fig. 2B). The absence of TH from wild-type SH-SY5Y cells is consistent with findings by other groups [26].

hTH1 and hTH2 were expressed at similar levels in the transfected SH-SY5Y cells (Fig. 2A). hTH1 and hTH2 were found to migrate at approximately 60 kDa. hTH1 and hTH2 can be distinguished as two different sized bands when the gels are run their entire length (>10 cm). Similar separation of the two isoforms has previously been observed [15]. TH activity in the transfected cells was determined to be 0.23 ± 0.045 and 0.35 ± 0.042 nM DOPA produced/nM TH/min in hTH1 and hTH2 SH-SY5Y cells, respectively (Fig. 2B, no significant difference, $p > 0.05$). The expression of hTH1 or hTH2 in SH-SY5Y cells did not change cellular morphology (data not shown).

3.2. In situ phosphorylation of hTH1 and hTH2 at Ser19 and Ser40/44

The hTH1 and hTH2 SH-SY5Y cells were characterized with regard to the phosphorylation of the three main Ser residues in TH (Ser19, Ser31 and Ser40 in hTH1; Ser19, Ser35 and Ser44 in hTH2). hTH1 and hTH2 SH-SY5Y cells were stimulated with 10 μM forskolin for 5 min, and assayed for phosphorylation and TH protein levels as described in the Materials and methods section. Stimulation with forskolin significantly increased the phosphorylation of Ser40/44 in both hTH1 and hTH2 cells ($p < 0.01$, Fig. 3). There was no significant difference in the level of forskolin-induced phosphorylation of Ser40/44 between hTH1 and hTH2 cells. Ser31 phosphorylation levels were unchanged in hTH1 (Fig. 3), and phosphorylation of Ser35 was not detectable in hTH2 in either the control or stimulated cells (data not shown). Forskolin did not change Ser19 phosphorylation (Fig. 3) or total TH protein (data not shown) levels in either cell type.

hTH1 and hTH2 SH-SY5Y cells were stimulated with 10 μM muscarine for 5 min. Muscarine was found to significantly increase

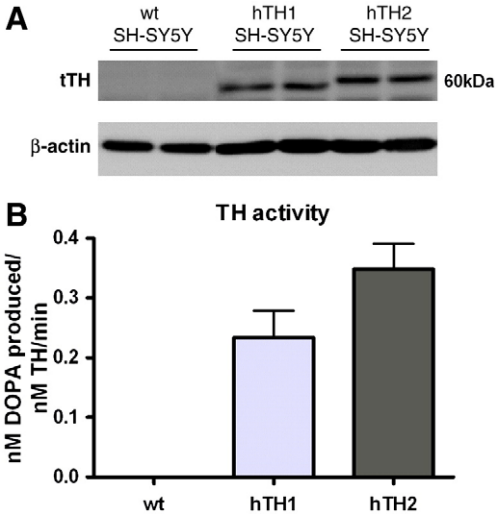

Fig. 2. Characterization of stably transfected SH-SH5Y cells. (A) Wild-type (wt), hTH1 and hTH2 SH-SY5Y cell extracts were analyzed for TH protein and β-actin content as described in the Materials and methods section. Results shown are representative. (B) Wild-type (wt), hTH1 and hTH2 SH-SY5Y cells were lysed and assayed for TH activity as described in the Materials and methods section. Activity is expressed as nM DOPA produced/nM TH/min + SEM ($n = 6$).

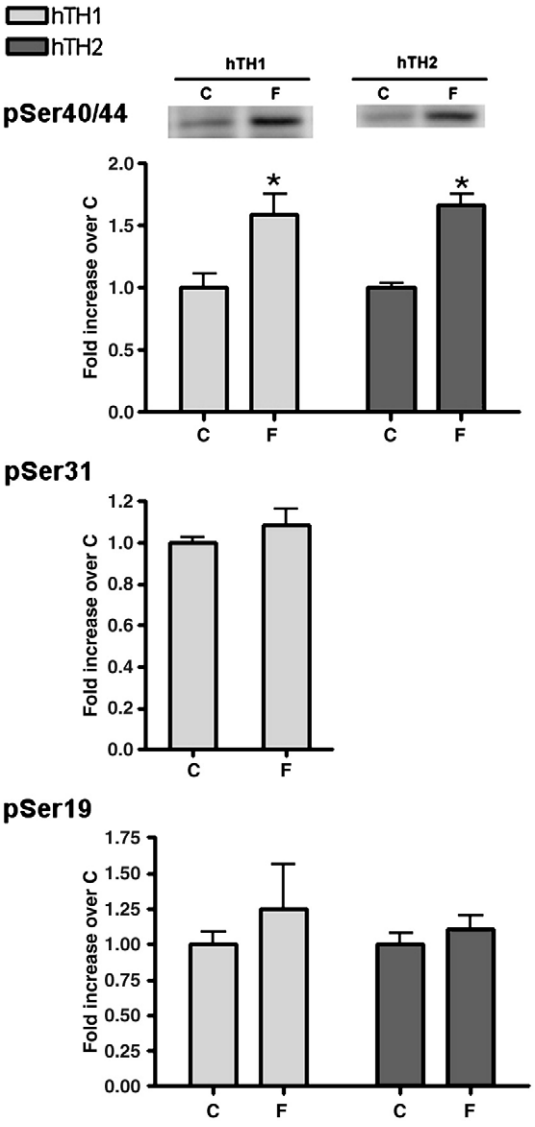

Fig. 3. Forskolin stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 10 μM foskolin (F) or the same volume of vehicle (C) for 5 min. TH phosphorylation levels were analyzed and quantified as described in the Materials and methods section. Representative pSer40 immunoblot is displayed above relative graph. Data are presented as the average fold increase relative to mean of control levels + SEM ($n = 8$ for C, $n = 5$ for F). *$p < 0.01$ vs. corresponding control.

the phosphorylation of Ser40/44 in both hTH1 and hTH2 cells ($p < 0.001$, Fig. 4). Muscarine also induced a substantial increase in the phosphorylation of Ser19 in both hTH1 and hTH2 cells ($p < 0.001$, Fig. 4). There was no significant difference in the level of muscarine-induced phosphorylation of Ser19 or Ser40/44 between hTH1 and hTH2 cells. There was no increase in the phosphorylation of Ser31 in hTH1 cells (Fig. 4), and again phosphorylation of Ser35 in hTH2 was not detectable in control or muscarine-stimulated cells (see representative immunoblot, Fig. 4). There was no change in TH protein levels in either cell type (data not shown).

3.3. In situ phosphorylation of Ser31 in hTH1, but not Ser35 in hTH2

hTH1 and hTH2 cells were stimulated with 50 ng/mL EGF for 30 min. EGF significantly increased the phosphorylation of Ser31 in hTH1 ($p < 0.05$, Fig. 5); however, no phosphorylation of Ser35 in hTH2 was detected (see representative immunoblot, Fig. 5). Stimulation with EGF did not result in any changes in the phosphorylation of Ser40/44 or Ser19 (Fig. 5), or TH protein levels (data not shown). EGF

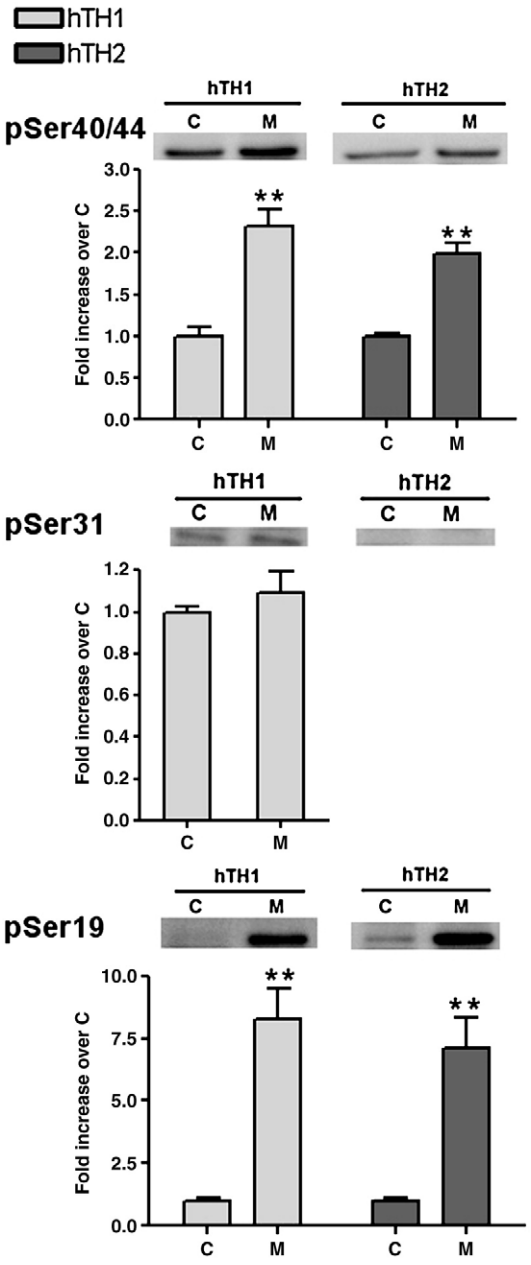

Fig. 4. Muscarine stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 10 μM muscarine (M) or the same volume of vehicle (C) for 5 min. TH phosphorylation levels were analyzed and quantified as described in the Materials and methods section. Representative pSer40/44, pSer31/35 and pSer19 immunoblots are displayed above relative graphs. Data are presented as the average fold increase relative to mean of control levels + SEM ($n=8$ for C, $n=5$ for M). **$p<0.001$ vs. corresponding control.

increased the phosphorylation of ERK in both hTH1 and hTH2 cells (data not shown).

3.4. Basal stoichiometry of serine phosphorylation in hTH1 and hTH2

The basal stoichiometry of serine phosphorylation was assessed using recombinant hTH standards phosphorylated to full stoichiometry as described in the Materials and methods section. The basal level of phosphorylation of hTH1 at Ser40 (0.15 mol phosphate/mol TH subunit) was significantly higher ($p<0.05$) than Ser44 phosphorylation in hTH2 (0.09 mol phosphate/mol TH subunit) (Fig 6). However, there was no significant difference between the basal level of phosphorylation of Ser19 in hTH1 and hTH2 (0.08 and 0.10 mol phosphate/mol TH subunit). Ser31 phosphorylation stoichiometry in hTH1 was determined to be 0.04 mol phosphate/mol TH subunit, while basal phosphorylation of Ser35 in hTH2 was not detectable.

3.5. pSer31-dependent hierarchical phosphorylation contributes to basal pSer40 levels in hTH1, but not hTH2 cells

We could not detect any phosphorylation of Ser35 in hTH2 under basal conditions or using stimuli that increased the phosphorylation of Ser31 in hTH1. Additionally, basal Ser40 phosphorylation stoichiometry was shown to be higher in hTH1 than Ser44 phosphorylation in hTH2. Phosphorylation of Ser31 can potentiate the phosphorylation of Ser40 in vitro [13]. We therefore decided to inhibit kinases that are known to contribute to Ser31 phosphorylation and determine the effect on Ser40/44 phosphorylation to see if phosphorylation of Ser31

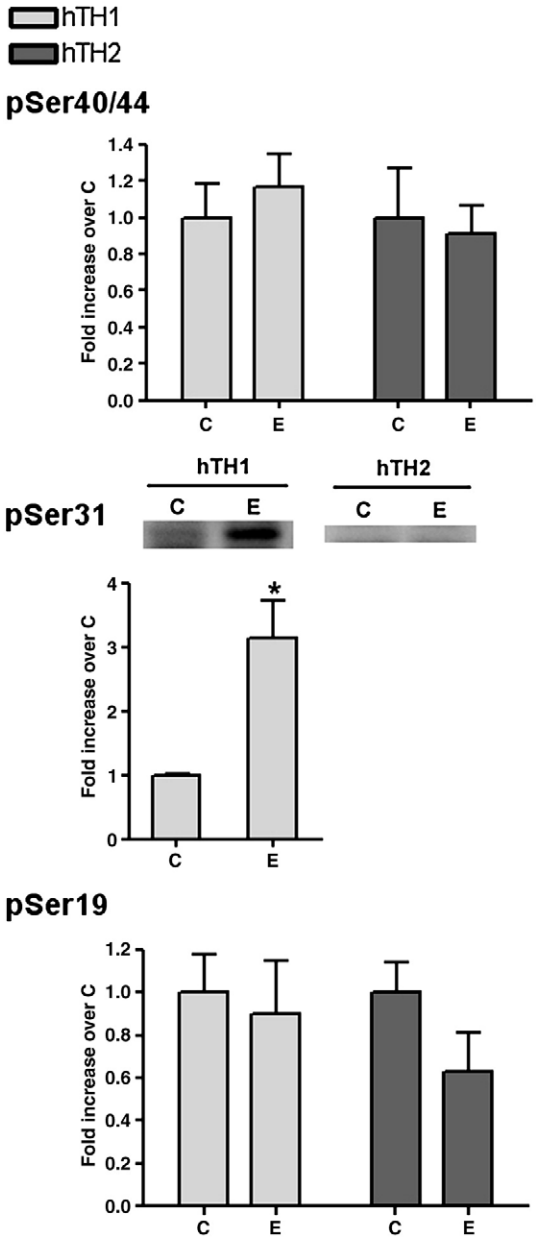

Fig. 5. EGF stimulation of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were stimulated with 50 ng/mL EGF (E) or the same volume of vehicle (C) for 30 min. TH phosphorylation levels were analyzed and quantified as described in the Materials and methods section. Representative pSer31/35 immunoblot is displayed above relative graph. Data are presented as the average fold increase relative to mean of control levels + SEM ($n=4$). *$p<0.05$ vs. corresponding control.

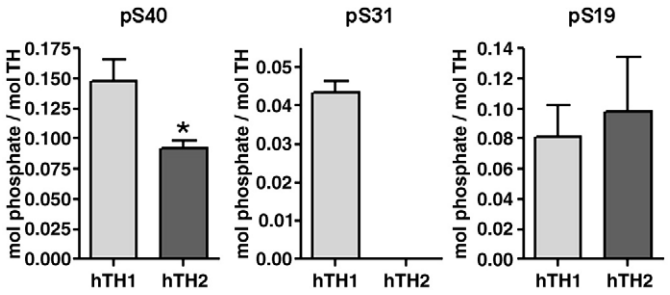

Fig. 6. Basal phosphorylation stoichiometry of hTH1 and hTH2 from SH-SY5Y cells. Stoichiometry of Ser phosphorylation in untreated cells extracts was determined by analyzing TH phosphorylation and TH protein levels alongside a series of recombinant TH standards phosphorylated to full stoichiometry at relative Ser residues. Data are presented as the average mol phosphate/mol TH subunit + SEM ($n=6$ for pS40 and pS19, $n=5$ for pS31). *$p<0.05$ for hTH1 vs. hTH2.

was contributing to the phosphorylation of Ser40 in a hierarchical manner. hTH1 and hTH2 SH-SY5Y cells were incubated for 30 min with 10 μM UO126, which inhibits MEK, a kinase upstream of ERK1/2. UO126 significantly inhibited the phosphorylation of ERK in both hTH1 and hTH2 cells ($p<0.001$, Fig. 7). UO126 significantly inhibited basal Ser31 phosphorylation in hTH1 cells ($p<0.05$, Fig. 7); Ser35 phosphorylation in hTH2 cells was not detectable (data not shown). UO126 also significantly inhibited the basal phosphorylation of Ser40 in hTH1 ($p<0.05$), but did not inhibit the phosphorylation of Ser44 in hTH2 (Fig. 7). Ser19 phosphorylation levels and total TH levels were not significantly decreased following treatment with UO126. These data indicate that phosphorylation of Ser31 can contribute to Ser40 phosphorylation in hTH1; however, this effect is absent in hTH2 cells due to the lack of Ser35 phosphorylation.

4. Discussion

Phosphorylation of Ser31 plays an important role in the regulation of hTH1, primarily by its effect on the potentiation of Ser40 phosphorylation. However, there has been no kinase identified that is capable of phosphorylating hTH2 at Ser35 in vitro. In this study, we have demonstrated that while basal phosphorylation of Ser40 in hTH1 is potentiated by the phosphorylation of Ser31 in situ, hTH2 is not subject to the same regulatory mechanism. In addition, we have demonstrated using stimuli that induce a large increase in Ser31 phosphorylation in hTH1, and also under conditions that increase intracellular Ca^{2+} mobilization, that there is no detectable phosphorylation of Ser35 in hTH2. This represents a fundamental difference between the regulatory mechanisms utilized by the major human TH isoforms.

To date, there have been no studies that have investigated the different phosphorylation profiles of hTH1 and hTH2 in situ. hTH1 and hTH2 were stably expressed in SH-SY5Y cells, a neuroblastoma cell line with a dopaminergic phenotype. Stimulation with forskolin induces a selective increase in the phosphorylation of Ser40/44 in hTH1 and hTH2 cells. Stimulation of hTH1 and hTH2 cells with muscarine results in an increase in the phosphorylation of both Ser19 and Ser40/44. This correlates with in vitro data demonstrating that the two major hTH isoforms are phosphorylated to an equal extent at Ser19 and Ser40/44 [11,27].

Stimulation of hTH1 cells with EGF resulted in a substantial increase in the phosphorylation of Ser31, which was not accompanied by an increase in Ser40 phosphorylation. It was expected that phosphorylation of Ser31 would potentiate the phosphorylation of Ser40. However, in the transfected SH-SY5Y cells, forskolin only induced a mild (1.6-fold) increase in the phosphorylation of Ser40,

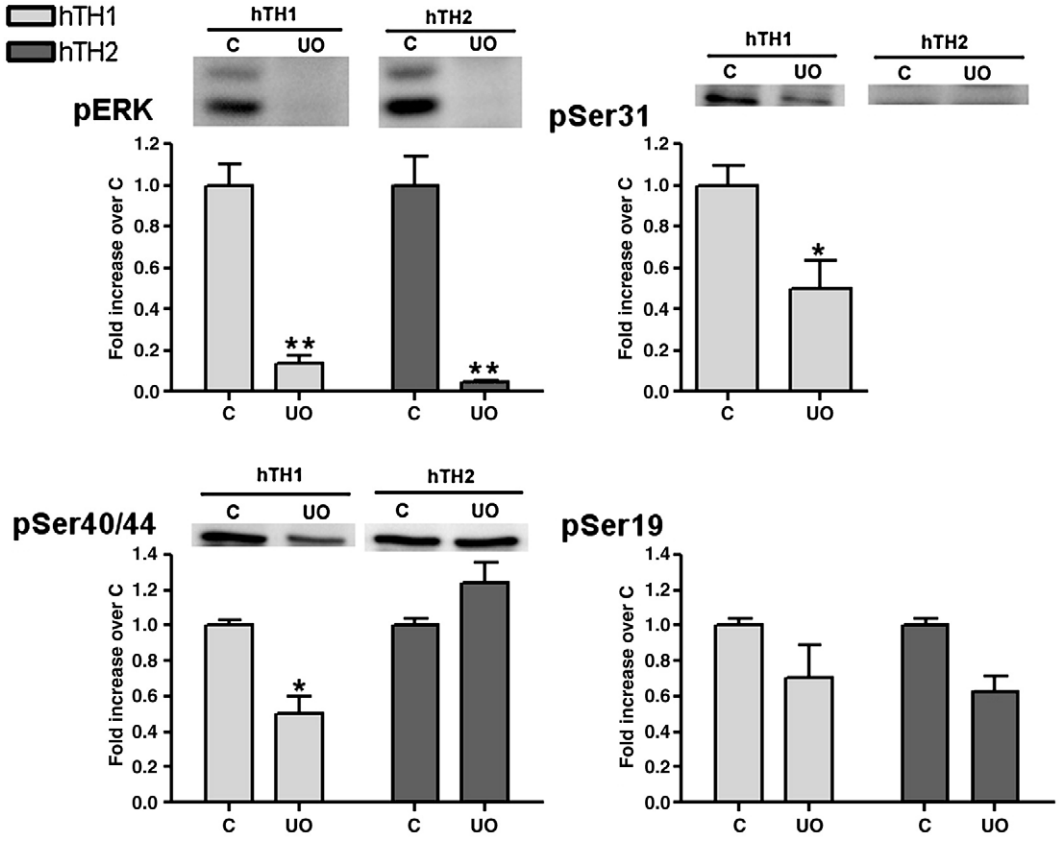

Fig. 7. UO126 treatment of hTH1 and hTH2 SH-SY5Y cells. SH-SY5Y cells were pre-incubated with 10 μM UO126 (UO) or the same volume of vehicle (C) for 30 min. Phosphorylated ERK (pERK) and TH phosphorylation levels were analyzed and quantified as described in the Materials and methods section. Data are presented as the average fold increase relative to mean of control levels + SEM ($n=5$, $n=6$ for pERK). *$p<0.05$ vs. corresponding control, **$p<0.001$ vs. corresponding control.

which is much smaller than the approximately 4-fold forskolin-induced increase in Ser40 phosphorylation that was previously found in bovine adrenal chromaffin cells [13,14]. To further examine this, the stoichiometry of basal phosphorylation levels of all three Ser residues was determined. It was shown that the basal phosphorylation of Ser40 was significantly higher than basal phosphorylation of Ser44 in hTH2. In addition, the basal phosphorylation levels of both Ser40 and Ser44 in hTH1 and hTH2, respectively, were 3–5-fold higher than the basal phosphorylation of Ser40 in chromaffin cells [14], and also higher than the basal Ser40 phosphorylation in PC12 cells [28,29]. However, the basal phosphorylation of Ser19 was similar in hTH1 and hTH2 cells, and was similar to that previously determined in chromaffin and PC12 cells [14,28,29]. The basal phosphorylation stoichiometry of Ser31 in hTH1 was also found to be similar to that determined previously in chromaffin cells [13]. When viewed together, this may mean that there is a higher basal level of Ser40/Ser44 kinase activity in the transfected SH-SY5Y cells, and could explain why phosphorylation of Ser31 alone could not further increase Ser40 phosphorylation. In effect, we may have already exceeded the level of Ser40 phosphorylation that can be altered by hierarchical phosphorylation via Ser31.

As previously noted, there is a significantly higher level of basal phosphorylation of Ser40 in hTH1 than Ser44 in hTH2. To examine whether Ser31 phosphorylation was contributing to the higher level of basal phosphorylation of Ser40 in hTH1 in situ, we treated the cells with UO126 which significantly inhibited the phosphorylation of ERK in both hTH1 and hTH2 cells, and resulted in a 50% inhibition of Ser31 phosphorylation in hTH1. Importantly, there was a 50% inhibition of Ser40 phosphorylation in hTH1 that was not accompanied by a concomitant inhibition of Ser44 phosphorylation in hTH2. The inhibition of Ser40 phosphorylation in hTH1 cells must be due to an UO126-mediated inhibition of Ser31 phosphorylation; if UO126 was acting upstream of the Ser40 kinase pathway, then a decrease in phosphorylation of Ser40/44 would be present in both cell types. Therefore, we have demonstrated that while hTH1 is regulated by Ser31-mediated hierarchical phosphorylation of Ser40 under basal conditions in situ, hTH2 is not subject to this form of regulation.

While EGF induced a substantial increase in the phosphorylation of Ser31 in hTH1, it did not increase the phosphorylation of Ser35 in hTH2. Activation of the muscarinic receptor results in the mobilization of Ca^{2+} from intracellular stores and activation of Ca^{2+}-dependent kinases such as CaMKII [30,31]. Muscarine potently increased the phosphorylation of Ser19 and Ser40/44 in both hTH1 and hTH2, which is consistent with the activation of Ca^{2+}-dependent kinases; however, stimulation with muscarine for 5 min (Fig. 5) or 30 min (data not shown) was not able to increase Ser31/35 phosphorylation in hTH1 or hTH2 cells. Therefore, using a range of stimuli that activate a variety of intracellular signaling pathways, we have not detected Ser35 phosphorylation in hTH2 in situ. This correlates with in vitro data, where there has been no kinase identified that is capable of reproducibly phosphorylating hTH2 at Ser35. It is likely that the 4 amino acid substitution in hTH2 is altering the ability of kinases to recognize and phosphorylate the Ser35 residue.

This is the first study to demonstrate that hTH1 and hTH2 are differentially regulated in situ. The lack of phosphorylation of Ser35 in hTH2 in situ means that Ser35 is not able to facilitate the hierarchical phosphorylation of Ser44. Haycock and colleagues have demonstrated that the human TH isoforms may be differentially distributed in different cell populations. For example, in dopaminergic cells of the substantia nigra, it was shown that although all four human TH isoforms were expressed in the cell body, hTH1 was selectively distributed along the axons and terminal fields of these neurons [20]. The selective targeting of the isoforms would provide a mechanism for the region-specific modulation of TH activity. hTH1 and hTH2 are subject to differential regulation, and therefore the rate of catecholamine synthesis that occurs at specific cellular regions would depend on the relative amounts of the isoforms that are present in that region. Therefore, the differential regulation of the major human TH isoforms may have important consequences for the control over catecholamine biosynthesis in vivo.

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