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Research paper

Tetramethylpyrazine induces SH-SY5Y cell differentiation toward the neuronal phenotype through activation of the PI3K/Akt/Sp1/TopoIIβ pathway

Yong-xin Yan[a], Jun-xia Zhao[a], Shuo Han[b], Na-jing Zhou[a], Zhi-qiang Jia[a], Sheng-jie Yao[a], Cui-li Cao[b], Yan-ling Wang[a], Yan-nan Xu[a], Juan Zhao[a], Yun-li Yan[a,*], Hui-xian Cui[b,c]

[a] Department of Cell Biology, Hebei Medical University, Hebei, PR China
[b] Department of Human Anatomy, Hebei Medical University, Hebei, PR China
[c] Hebei Key Laboratory for Brain Aging and Cognitive Neuroscience, Hebei, PR China

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ABSTRACT

Tetramethylpyrazine (TMP) is an active compound extracted from the traditional Chinese medicinal herb Chuanxiong. Previously, we have shown that TMP induces human SH-SY5Y neuroblastoma cell differentiation toward the neuronal phenotype by targeting topoisomeraseIIβ (TopoIIβ), a protein implicated in neural development. In the present study, we aimed to elucidate whether the transcriptional factors specificity protein 1 (Sp1) and nuclear factor Y (NF-Y), in addition to the upstream signaling pathways ERK1/2 and PI3K/Akt, are involved in modulating TopoIIβ expression in the neuronal differentiation process. We demonstrated that SH-SY5Y cells treated with TMP (80 μM) terminally differentiated into neurons, characterized by increased neuronal markers, tubulin βIII and microtubule associated protein 2 (MAP2), and increased neurite outgrowth, with no negative effect on cell survival. TMP also increased the expression of TopoIIβ, which was accompanied by increased expression of Sp1 in the differentiated neuron-like cells, whereas NF-Y protein levels remained unchanged following the differentiation progression. We also found that the phosphorylation level of Akt, but not ERK1/2, was significantly increased as a result of TMP stimulation. Furthermore, as established by chromatin immunoprecipitation (ChIP) assay, activation of the PI3K/Akt pathway increased Sp1 binding to the promoter of the TopoIIβ gene. Blockage of PI3K/Akt was shown to lead to subsequent inhibition of TopoIIβ expression and neuronal differentiation. Collectively, the results indicate that the PI3K/Akt/Sp1/TopoIIβ signaling pathway is necessary for TMP-induced neuronal differentiation. Our findings offer mechanistic insights into understanding the upstream regulation of TopoIIβ in neuronal differentiation, and suggest potential applications of TMP both in neuroscience research and clinical practice to treat relevant diseases of the nervous system.

1. Introduction

Tetramethylpyrazine (TMP), also called *ligustrazine*, is a biologically active alkaloid isolated from the traditional herbal medicine *Ligusticum wallichii Franch*. It has long been used in clinical practice to improve circulation and prevent clot formation (Xu et al., 2003). It is well known that TMP acts as a calcium channel antagonist (Pang et al., 1996) or as an antioxidant (Yang et al., 2008). Recently, TMP has received attention for its distinctive roles in stimulating neurogenesis after focal ischemia in the rat brain (Xiao et al., 2010), inducing neuronal differentiation of rat neural stem cells (Tian et al., 2010) and displaying neuro-protective roles in traumatic spinal cord injury (Hu et al., 2013) and chronic hypoxia of the medulla oblongata in a rat model (Ding et al., 2013), in addition to improving scopolamine-induced memory impairment (Wu et al., 2013). These results suggest TMP as a promising candidate for therapy in relevant neurologic disorders. However, the molecular mechanism underlying TMP's functional roles in the human nervous system, especially TMP's stimulus of neurogenesis or neuronal differentiation, needs to be further investigated.

Abbreviations: ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ERK1/2, extracellular signal-regulated kinase 1/2; LDH, lactate dehydrogenase; MAP2, microtubule associated protein 2; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny tetrazolium bromide; NF-Y, nuclear factor Y; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase; Sp1, specificity protein 1; TBS, Tris-buffered saline; TMP, tetramethylpyrazine; TopoII, topoisomeraseII.

* Corresponding author at: Department of Cell Biology, Institute of Basic Medicine, Hebei Medical University, 361 Zhongshan East Road, Shijiazhuang 050017, China.
E-mail address: yanyunli2015@163.com (Y.-l. Yan).

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DNA topoisomerasesII (TopoII) are ATP-dependent enzymes that alter DNA topology by cleavage and re-ligation of the DNA. Eukaryotic TopoII exists as two isoforms named TopoIIα and TopoIIβ (Austin and Marsh, 1998). They are encoded by the TOP2A and TOP2B genes located on chromosomes 17q21 ± 22 and 3p24, respectively (Forterre et al., 2007; Tan et al., 1992). Although both enzymes are highly homologous, they are genetically distinct and exhibit different patterns of expression and cellular function. Expression of TopoIIα is cell cycle-dependent, and essential for DNA replication and chromosome segregation in mitotic cells (Turley et al., 1997; Wang, 2002), whereas TopoIIβ levels remain unchanged during cell cycle progression, and are maximal in terminally differentiated tissues (Turley et al., 1997; Vávrová and Šimůnek, 2012). Recent studies have determined that TopoIIβ is a critical molecule in neuronal differentiation (Isik et al., 2015; Tsutsui et al., 2001). It plays a pivotal role in neural development (Yang et al., 2000) and axon guidance (Nur-E-Kamal et al., 2007). The transcriptional induction that is dependent on TopoIIβ may be specific to neuronal genes (Heng and Le, 2010; Lyu et al., 2006). Moreover, it has been shown that TopoIIβ regulates transcription of neuronal genes by directly binding to their regulatory regions, and emerged as an epigenetic regulator of transcription in later-stage neural development (Lyu et al., 2006; Tiwari et al., 2012).

The transcription of the TopoIIβ gene is regulated mainly by a region between −553 and −481 relative to the transcription start site, with binding sites for specificity protein 1 (Sp1) and nuclear factor Y (NF-Y) on the gene promoter (Lok et al., 2002). In previous studies, we have reported that Sp1 regulates TopoIIβ expression in SH-SY5Y cells during retinoic acid-induced neuronal differentiation (Guo et al., 2014). We also demonstrated that TMP promotes SH-SY5Y cell differentiation via enhanced TopoIIβ expression, which is mediated by transcriptional activation of TopoIIβ through increased association of acetylated histones H3 and H4 with the TopoIIβ gene promoter. These epigenetic alterations on the TopoIIβ gene structure may lead to an open chromatin state for transcription factor access (Yan et al., 2014). However, whether TMP induces neuronal differentiation of SH-SY5Y cells via Sp1 or NF-Y to regulate TopoIIβ gene expression, as well as related upstream signaling molecules, need to be clearly determined.

Currently, accumulating evidence has indicated that neuronal differentiation is regulated by several cell signaling molecules, including phosphatidylinositol 3-kinase (PI3K)/Akt (Chan et al., 2013; Lopez-Carballo et al., 2002) and the extracellular signal-regulated protein kinase 1/2 (ERK 1/2) pathways (Tian et al., 2010; Tsao et al., 2013). ERK1/2 is considered to be able to induce neurogenesis by regulating certain genes mediated by Sp1 (Dore et al., 2009), and the PI3K/Akt/Sp1 pathway is also positively correlated to cellular differentiation (Takao et al., 2012; Yin et al., 2012). Moreover, previous studies have shown that TMP impacts on different cellular functions through both the PI3K/Akt and ERK1/2 pathways (Lv et al., 2012; Tian et al., 2010; Zhang et al., 2014). Recently, direct evidence has shown that the ERK1/2 signaling pathway is involved in TMP-induced differentiation of rat neural stem cells into neurons (Tian et al., 2010).

To unravel the relationship between these signaling molecules and TopoIIβ gene expression during TMP-induced neuronal differentiation, the present study used human SH-SY5Y neuroblastoma cells as a differentiating model. Following 3–5 days' treatment with TMP (80 μM), the cells were shown to terminally differentiate into neurons through upregulation of TopoIIβ. PI3K/Akt signaling, but not ERK1/2, is required for this differentiation process. We further demonstrated a positive connection between the PI3K/Akt signal and the increased expression of TopoIIβ mediated by Sp1, suggesting that TMP induces neuronal differentiation through interactions with the PI3K/Akt/Sp1/TopoIIβ signaling pathway. In contrast to Sp1, NF-Y was found to be independent of both PI3K/Akt and ERK1/2 signals, and levels remained unchanged following the differentiation process. Unexpectedly, using chromatin immunoprecipitation (ChIP), we found increased NF-Y binding to the TopoIIβ promoter. The possible role of NF-Y in transcription of the TopoIIβ gene during TMP induction of neuronal differentiation is also discussed in this study.

2. Materials and methods

2.1. Regents and antibodies

TMP was purchased from Sigma (St. Louis, MO, USA) and was dissolved in dimethylsulfoxide (DMSO; Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The chemical structure of TMP is shown in Fig. 1A. The cell proliferation assay kit 3-(3,4-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Roche Diagnostics (Mannheim, Germany). We purchased nuclear dye 4′,6-diamidino-2-phenylindole (DAPI) from Sigma (St. Louis, MO, USA). The fetal bovine serum (FBS), 0.25% trypsin-EDTA, and a penicillin/streptomycin mixture were obtained from Gibco-BRL (Grand Island, NY, USA). Cell cycle analysis kits were from Roche Applied Science (Indianapolis, IN, USA). We purchased rabbit anti-cleaved-caspase-3, anti-phospho-Akt (Ser473) and anti-phospho-ERK1/2 (Thr202/Tyr204) antibodies from Cell Signaling (Boston, MA, USA). We purchased rabbit anti-Akt (Ser473) antibody from Epitomics (Burlingame, CA, USA). We purchased rabbit anti-MAP2, rabbit anti-β-actin, rabbit anti-tubulin βIII, rabbit anti-TopoIIα, rabbit anti-TopoIIβ and rabbit anti-ERK 1/2 antibodies from Bioworld Technology, Inc. (Minneapolis, MN, USA). Rabbit anti-Sp1 antibody was obtained from Abcam (Cambridge, MA, USA). Rabbit anti-NF-YA antibody and all secondary antibodies against mouse and rabbit IgG were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). MEK inhibitor U0126 was purchased from Calbiochem (San Diego, CA, USA). PI3K inhibitor LY294002 was purchased from Calbiochem (Merck, Germany). They were all diluted in DMSO. LDH Cytotoxicity Assay Kits and ChIP assay kits were obtained from Beyotime Biotechnology Institute (Shanghai, China). The eukaryotic protein synthesis inhibitor cycloheximide (CHX) was obtained from Sigma Chemical Co. (St. Louis, MO, USA). The catalytic inhibitor of TopoIIβ ICRF-193, bis (2,6-dioxopiperazine) and the RNA polymerase II inhibitor α-amanitin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

2.2. Cell culture and differentiation induction

SH-SY5Y Cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 units/mL of penicillin and 100 μg/mL of streptomycin in a 37 °C humidified incubator with an atmosphere of 5% CO_2 in air. For promotion of neuronal differentiation, the cells were cultured with an inducing medium composed of 80 μM TMP in DMEM with 3% FBS as described in previous studies (Tian et al., 2010; Yan et al., 2014), and an equal volume of DMSO was added for the control cells. The control and TMP-treated cells were cultured for 5 days, changing the fresh medium every two days. Except for the TMP exposure, all control cells were handled in parallel with the test cells.

2.3. Cell viability measured by MTT assay

SH-SY5Y cells in the logarithmic growth phase were seeded in a 96-well culture plate with 1×10^4 cells/well. Twenty-four hours after plating, 0, 10, 20, 40, 80 and 120 μM TMP were used respectively to treat cells and growth was evaluated on days 0 to 5. At each time point, 20 μL MTT (5 mg/mL) was added into the 200 μL