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Tetramethylpyrazine promotes proliferation and differentiation of neural stem cells from rat brain in hypoxic condition via mitogen-activated protein kinases pathway in vitro

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ABSTRACT

This study investigated the effects of tetramethylpyrazine (TMP), an active element of traditional Chinese medicine Ligusticum Chuanxiong, on proliferation and differentiation of neural stem cells (NSCs) from rat brain in hypoxia condition and the activation of mitogen-activated protein kinases (MAPKs) signaling pathway during the processes. The results showed that TMP promoted the proliferation and differentiation of the NSCs into neurons. TMP increased the phosphorylation of ERK1/2 and decreased the phosphorylation of p38 at different time points. ERK inhibitor (U0126) in part blocked the differentiation of the NSCs into neurons induced by TMP. Our findings demonstrated that TMP enhanced the proliferation and differentiation of NSCs of rat after hypoxia in vitro, in which the phosphorylation of ERK and p38 was involved.

Tetramethylpyrazine (TMP), a pure compound derived from Ligusticum chuanxiong (LC), which is widely used in the treatment of ischemic stroke [14] and has been reported to possess a diverse array of pharmacological functions in the modulation of arterial resistance, cerebral blood flow, platelet function, microcirculation, and capillary permeability [7,12,18,20].

Neural stem cells (NSCs) have the capacity for generating new neurons and glial supporting cells and are present in adult special brain area. Widespread neurogenesis and gliogenesis occur in adult mammalian nervous system in response to certain stress conditions, including hypoxia, and the injuries with loss and the death of a large number of neurons can activate the regeneration process of NSCs. Experimentally, TMP prevents hypoxic and excitotoxic cell damage in hippocampal neurons and experimental animals [4,11,19]. TMP promotes NSCs proliferation in ependymal/subventricular zone and differentiation into neurons with decreased nNOS expression in rat with focal cerebral ischemia [23]. Most of the research focuses on the direct neuroprotection on the neurons and antiapoptotic effect of TMP in vivo [11,19]. But it is unclear about what roles it plays and through which signal pathway it plays a role in the differentiation of NSCs under hypoxic condition in vitro.

The members of the mitogen-activated protein (MAP) kinase family, including extracellular signal-related protein kinases (ERKs), stress-activated kinases, c-Jun NH2-termianl kinases (JNKs) and p38 MAP kinases, regulate neural cell proliferation and differentiation programs. JNK and p38 MAP kinase, play an important role in development and differentiation of central nervous system (CNS). A recent study demonstrated that EGF induced mouse ES cell proliferation by phosphorylation of Cx43, which was mediated by Ca2+/PKC, p44/42 and p38 MAPKs pathways [16]. Using cultured post-natal hippocampal stem cells as a model, Hu et al. demonstrated that NT3 stimulated NSCs to differentiate into oligodendrocyte precursors (OLPs) through an extracellular Erk1/2-dependent pathway [8]. But little is known about the change of phophorylation of MAPK signaling molecules in NSCs of rat after TMP-treatment.

We have reported that focal cerebral ischemia stimulates neurogenesis in rat [23] and demonstrated that TMP could protect ischemic brain from damage, and promote cell proliferation and differentiation stimulated by ischemia in vivo [17]. In this study, we investigated the effects of TMP on cell proliferation and differentiation of NSCs of rat, and whether these effects are mediated by MAPK pathways in hypoxic culture conditions.

NSCs from cortex of embryonic day 15 (ED15) Sprague–Dawley fetal rats were isolated and cultured (Experimental Animal Center of Xi'an Jiaotong University College of Medicine). All animals' care complied with the National Institutes of Health guide for the care and use of laboratory animals. Briefly, fresh cortex were

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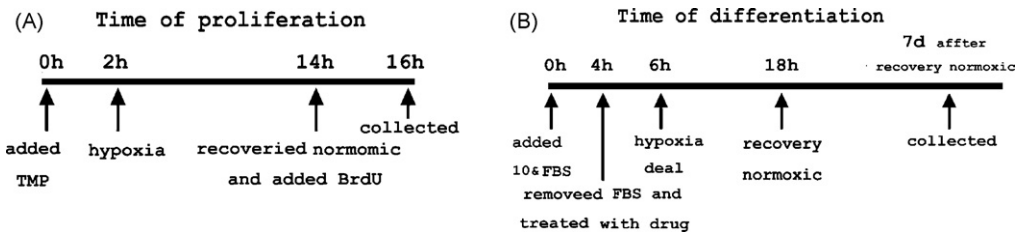

Fig. 1. Experimental protocol for study of proliferation [A] and differentiation [B].

dissociated from ED15 embryos and incubated in a digestion solution (trypsin 0.01%, EDTA 200 μM, glucose 0.6%, $MgCl_2$ 1 mM) [3]. The cells were seeded at a density of 1.5×10^4 cells/ml in a mixture of serum-free DMEM/F12 (1:1) (basal medium) supplemented with 2% B27 supplement, 1% N2 supplement, 10 ng/ml bFGF and 2.5 μg/ml heparin (complete medium). The cells proliferated and formed primary neurospheres (about 80–100 μm in diameter) after 3 d of culture. The primary neurospheres were passaged after 5–7 d with compound digestion solution. The single cells were resuspended in serum-free medium and cultured for 3–5 d (passage 1 neurospheres).

NSCs dissociated from passage 1 neurospheres were used in the experiments. For cell proliferation study, NSCs from primary neurospheres were seeded at a density of 1.5×10^4 cells/ml and cultured for 3–5 d when passage 1 neurospheres formed. Then, the cells were treated with drugs and cultured in the complete medium for 16 h. For the differentiation study, cells from passage 1 neurospheres were seeded at a density of 1×10^4 cells/ml in 24-well plate, and were then cultured in basal medium with 10% FBS. After cultured for 4 h, the medium was replaced by complete medium to allow differentiation, the drug was given and the cells were cultured for 7 d. Fig. 1A and B shows the outline of the experimental procedures.

To induce hypoxia, the passage 1 NSCs were placed in a modular chamber with hypoxic condition of humidified 0.3%O_2, 94.7% N_2 and 5% CO_2 (BUGBOX, Ruskinn Technology). The cultures were performed in the hypoxic condition for 12 h, and then back to the normoxic condition for 3 d or 7 d.

Cells were seeded in 96-well plates (1×10^4). After 24 h, the cells were pretreated with different concentrations of TMP (1 μg/ml, 10 μg/ml, 30 μg/ml) (WuXi No. 7 Pharmaceutical Company, China) respectively for indicated time, and were then washed twice with PBS before 100 μl of 0.5 mg/ml MTT (Sigma, USA) was added in PBS.

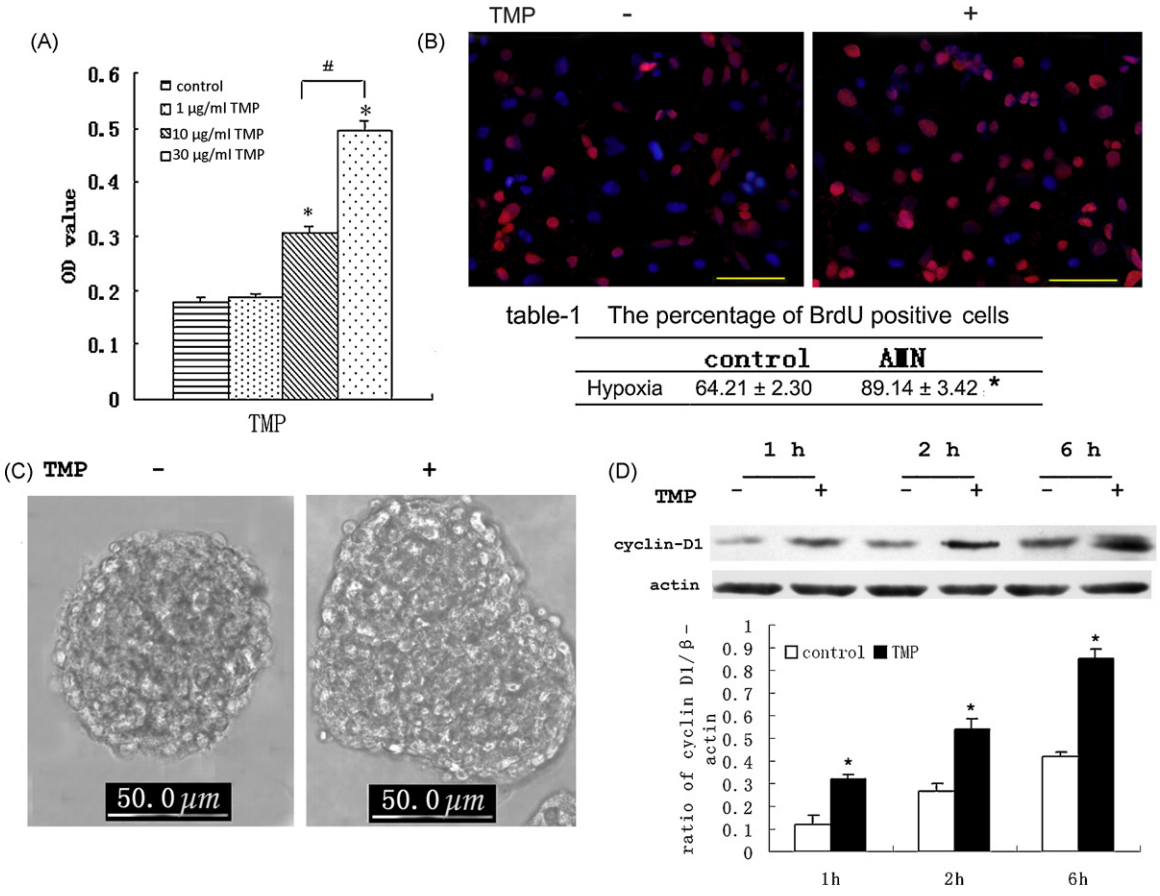

Fig. 2. Effect of TMP on proliferation of NSCs in hypoic condition. (A) OD value of the MTT assay reflects the cell proliferation with or without treatment of TMP. *Compared to the control: $p < 0.05$; (B) double staining of BrdU (red) and Hoechst 33342 (blue) in the merged images shows more proliferated NSCs in TMP-treated group. Scale bar = 50 μm; Table 1: the data are expressed as the percentage of the total cell population (means ± SEM) from four individual culture dishes (mean value was obtained from three random microscopic fields per dish) from two independent experiments. (C) The diameter of neurospheres in TMP group was greater. (D) Immunoblot analysis showed that cyclin D1 expression significantly increased at 1 h, 2 h, and 6 h after TMP-treatment. *p, #$p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).