Tetramethylpyrazine Ameliorated Hypoxia-Induced Myocardial Cell Apoptosis via HIF-1α/JNK/p38 and IGFBP3/BNIP3 Inhibition to Upregulate PI3K/Akt Survival Signaling

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Abstract:

Background: Hemorrhagic shock (HS) is the major cause of death from trauma. Hemorrhagic shock may lead to cellular hypoxia and organ damage. Our previous findings showed that HS induced a cardiac apoptosis pathway and synergistically caused myocardial cell damage in diabetic rats under trauma-induced HS. Tetramethylpyrazine (TMP) is a major biologically active ingredient purified from the rhizome of *Ligusticum wallichii* (called Chuang Xiong in Chinese). Chuan Xiong rescued cells from synergistic cardiomyoblast cell injury under high-glucose (HG) conditions plus hypoxia. TMP is one of the most important active ingredients that elevated the survival rate in ischemic brain injury and prevented inducible NO synthase expression to have anti-inflammatory effects against cell damage in different cell types.

Method: Here, we further investigate whether TMP can protect against hypoxic (<1% oxygen) conditions in H9c2 cardiomyoblast cells for 24 hrs. Results: Our results showed that hypoxia mediated through HIF-1α/JNK/p38 activation significantly elevated the levels of the hypoxia-related proteins HIF-1α, BNIP3 and IGFBP3, further enhanced the pro-apoptotic protein Bak and upregulated downstream Caspase 9 and 3, resulting in cell death. All of these phenomena were fully recovered under TMP treatment. We observed that TMP exerted this effect by...
activating the IGF1 receptor survival pathway, dependent primarily on PI3K/Akt. When PI3K (class I) was blocked by specific siRNA, the hypoxia-induced activated caspase 3 and cell apoptosis could not be reversed by TMP treatment. **Conclusion:** Our results strongly suggest that TMP could be used to restore hypoxia-induced myocardial cell apoptosis and cardiac hypoxic damage.

**Introduction**

A large number of people in Western society die due to myocardial ischemic injury resulting from coronary occlusion spectrum [1, 2]. Ischemic injuries, such as myocardial infarction and hemorrhage shock, are the terminus of heart disease. Myocardial infarction is a life-threatening heart injury that remains the major cause of mortality in industrialized countries [3-5]. In acute myocardial infarction, the myocardium is subjected to sudden ischemia due to blood supply interruption, causing sudden heart damage [3, 6]. Trauma-hemorrhage has been demonstrated to produce cardiac dysfunction via TNF-α levels, increasing during ischemia, which is associated with chronic heart failure, decreasing tissue perfusion, cellular hypoxia, organ damage and death [7, 8]. Hypoxic heart failure is a clinical syndrome defined as any condition where the rate of blood delivery to the organs and tissues is inadequate to supply their metabolic needs [9, 10]. Hypoxia-inducible factor-1α (HIF-1α) is a transcription factor that functions as the master regulator of gene expression in response to hypoxia. HIF-1α may function to regulate cell death or cell survival via downstream proteins such as heme oxygenase-1, inducible nitric oxide synthase, cyclooxygenase-2 and vascular endothelial growth factor [5, 11-16], the Bcl-2 family member Bcl-2/adenovirus E1B 19-kDa protein interacting protein 3 (BNIP3), and insulin growth factor binding protein 3 (IGFBP3) [13, 17-19]. This study identified HIF-1α as a key regulator of BNIP3 and IGFBP3 in cardiomyocytes. More evidence showed that BNIP3 activation of Bax/Bak opens the mitochondrial permeability transition pores and significantly induces mitochondrial dysfunction and cell death in I/R injury. Therefore, elucidation of how to restore hypoxic damage through BNIP3 and IGFBP3 suppression is of prime importance.

Studies have shown that IGF-I in the cardiovascular system is an important regulatory agent for maintaining cardiac functions. In humans, IGF-I appears to improve cardiac function after myocardial infarction and promotes tissue remodeling, and low IGF-I levels are associated with increased risk for myocardial infarction and heart failure. It has recently been reported that the pro-apoptotic factor Bad is phosphorylated and inactivated by IGF-1 through PI3K and Akt pathway [20, 21]. On the other hand, mitogen-activated protein kinases (MAPKs) are also activated during ischemia and may contribute to the structural and functional changes leading to apoptosis, fibrosis and heart failure [22]. Evidence has shown that p38 MAPK activation in cardiomyocytes induced TNF-α release, resulting in left ventricular (LV) remodeling marked by inflammation [23]. Our previous study found that the PI3K-Akt pathway is required for E2 and estrogen receptor-α to block LPS-upregulated JNK1/2 activation, IκB degradation, and NFκB, thereby protecting against LPS-induced myocardial cell damage. However, the regulatory mechanisms of hypoxia on IGF-1/PI3K/Akt and MAPKs in H9c2 cardiomyoblast cells have not been fully investigated.

In a previous study, we observed the anti-inflammatory and anti-apoptotic effects of Chuan Xiong on the liver cells in hemorrhagic shocked rats in our 150 herbal drug screening experiment. Chuan Xiong is widely applied in the treatment of attenuating free radicals via increasing the intracellular Ca²⁺ concentration and could also be a calcium antagonist to protect the rat myocardium during ischemia-reperfusion injury [24, 25]. It has also been reported that Ligusticum wallichii was used in relaxing the porcine coronary artery [26]. Tetramethylpyrazine (TMP) is a major biologically active ingredient purified from the rhizome of Ligusticum wallichii (called Chuan Xiong in Chinese) [27]. TMP was demonstrated to significantly elevate the survival rate and provide neuro-protection against
ischemic brain injury in rats [28]. TMP exerts a preventive effect against oxidative stress, myocardial injury, renal toxicity and hepatocellular injuries as well [29-32]. TMP has also been demonstrated to prevent the inflammation efficiency mediated inhibitory effect on the production of NO and iNOS in microglial cells and different types of cell types [33, 34]. The protection mechanisms of TMP in hypoxic heart tissue and whether TMP could act directly against hypoxic injuries in cardiomyocytes remain unclear. We therefore designed experiments to investigate the mechanism of TMP on hypoxia-induced myocardial injuries and further elucidated the mechanisms underlying the protective effects of TMP against this damage. We aim to identify the roles of HIF-1α and MAPKs during hypoxia and the amount of change in hypoxia-related BNIP3 and IGFBP3 proteins and IGF-1/PI3K/Akt signaling. We also aim to further understand if all hypoxia-induced phenomena could be fully recovered through TMP treatment mediated through the activated IGF1 receptor survival pathway and PI3K/Akt activities.

**Materials and Methods**

**Cell culture and reagents**

H9c2 cardiomyoblasts from the American Type Culture Collection (ATCC, CRL-1446) (Rockville, MD) were cultured in 100-mm or 60-mm culture dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1 mM HEPS buffer, and 10% Clontech fetal bovine serum in humidified air (5% CO2) at 37°C. H9c2 cells were incubated in serum-free essential medium overnight before treatment with the indicated agents. For hypoxia experiments, the H9c2 cardiomyoblast cells (ATCC) were cultured with 5% CO2, 94% N2, and 1% O2 at 37°C. The cells were incubated for 24 hrs under hypoxic conditions. TMP was purchased from Sigma (Sigma Chemical Co., St. Louis, MO).

**TUNEL Assay**

The TUNEL (TdT-mediated digoxigenin- dUTP nick-end labeling) method was carried out with a commercially available in situ apoptosis detection kit (Roche Molecular Biochemicals). To explore the effect of TMP on hypoxia-induced H9c2 apoptosis, staining was performed according to the manufacturer’s protocol. TUNEL-positive cells were identified with a fluorescent microscope using an excitation wavelength in the 450-500 nm range and a detection wavelength in the 515-565 nm range (green). The percentage of apoptotic cells was calculated by dividing the number of TUNEL-positive cells by the total number of cells visualized in the same field. Three digitized images with similar total cell numbers were selected from each cover slip for counting and averaging and were considered as one independent experiment. Three independent experiments were then averaged and statistically analyzed.

**Examination of protein expression by Western blotting**

A total of 5x10⁵ H9c2 cells were plated onto a 10-cm dish and incubated at 37°C for post-treatment with high glucose for 12 hrs and then combined into a hypoxic environment and co-treated with Chuan Xiong for 24 hrs. To isolate the total proteins, the H9c2 cells were washed with cold PBS and resuspended in lysis buffer (50 mM Tris, pH 7.5, 0.5 M NaCl, 1.0 mM EDTA, pH 7.5, 10% glycerol, 1 mM BME, 1% IGEPAL-630 and a proteinase inhibitor cocktail (Roche Molecular Biochemicals, Germany)). After incubation for 30 min on ice, the supernatant was collected by centrifugation at 12000 g for 15 min at 4°C. The protein concentration was determined using the Bradford method. Samples containing equal amounts of protein (50 μg) were loaded and analyzed using Western blot analysis. Briefly, the proteins were separated using 10% SDS-PAGE and transferred onto a PVDF membrane (Millipore, Belford, Massachusetts, USA). The membranes were blocked with blocking buffer (5% non-fat dry milk, 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for at least 1 hr at room temperature. The membranes were incubated with primary antibodies (caspase-3, HIF-1α, IGFBP3, Akt, Bcl-2, Bak, Bax and β-actin (Santa Cruz Biotechnology, Inc. Santa Cruz, California, USA), cleaved caspase-9, cleaved caspase-3, phosphorylated-Akt Ser473 (p-Akt) (Cell Signaling)), in the above solution on an orbital shaker at 4°C overnight. Following primary antibody incubation, the membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG).
Apoptosis assayed by annexin V and propidium iodide (PI) flow cytometry

H9c2 cells were plated at a density of 2×10^5 cells/well in 6-well plates. After exposure to the hypoxic environment and treatment with various concentrations of TMP (10 μM, 50 μM, and 100 μM) for 24 hrs, apoptotic cells were assayed with flow cytometry using the Annexin V-FITC Apoptosis Detection Kit (Biovision). The cells were suspended in 1X binding buffer and incubated with 5 μL annexin V and 5 μL propidium iodide (PI) for 15 min at 4°C in the dark. The cells were then analyzed by flow cytometry using the fluorescein isothiocyanate (FITC) signal detector (FL1) and the PI signal detector (FL2) in a FACSCalibur (BD, Bioscience, San Jose, CA).

siRNA transfection assay

Double-stranded siRNA sequences targeting HIF-1α, BNIP3, IGFBP3 and PI3K mRNAs were obtained from Sigma Biotechnology. The non-specific siRNA (scramble) was non-targeting. The cells were cultured in 60-mm well plates in medium. siRNA transfection was carried out with transfection reagent (PureFection™, System Biosciences, Mountain View, CA). Specific silencing was confirmed by immunoblotting with cellular extracts after transfection.

Neonatal cardiomyocyte primary culture

Neonatal cardiomyocytes were isolated and cultured using the commercial Neonatal Cardiomyocyte Isolation System Kit according to the manufacturer’s directions (Cellutron Life Technology, Highland Park, New Jersey, USA). Briefly, hearts from one- to two-day-old Sprague-Dawley rats were removed. The ventricles were pooled, and the ventricular cells dispersed using digestion solution at 37°C. Ventricular cardiomyocytes were isolated and cultured in DMEM containing 10% fetal bovine serum, 100 g/ml penicillin, 100 g/ml streptomycin, and 2 mM glutamine. After 3-4 days, the cells were incubated in serum-free essential medium overnight before treatment with the indicated agents.

Statistical analyses

Data are expressed as the mean ± S.E.M. Statistical evaluation was carried out using One-way Analysis of Variance (One-way ANOVA) followed by Scheffe’s multiple range test. P values less than 0.05 were considered to indicate a significant difference.

Results

TMP inhibited cell death induced by hypoxia in H9c2 cells

As we know, ischemia can cause cell death in cardiomyocytes [3]. We examined whether TMP could prevent hypoxia-induced H9c2 cell death. Therefore, we co-treated the cells with hypoxia and TMP (10, 50 and 100 μM) for 24 hrs. The results showed that TMP could significantly suppress hypoxia-induced apoptotic bodies from forming in a concentration-dependent manner (10 μM, 50 μM, and 100 μM) using the TUNEL assay in hypoxia-induced H9c2 cells (Fig. 1A). We analyzed the apoptotic ratio to confirm the above result using flow cytometry, as shown in Fig. 1B. TMP efficiently reduced the apoptosis ratio from hypoxia (6.9%) to 4.7% at 10 μM TMP, 3.3% at 50 μM TMP, and 3.4% at 100 μM TMP. Moreover, hypoxia exposure inhibited pro-apoptotic activities; p-IGF1R, p-PI3K, and p-Akt were fully restored by TMP, and the hypoxia-enhanced apoptotic proteins Bak and cleaved-caspase3 were completely blocked by TMP in a concentration-dependent manner. These results indicated that TMP could have an anti-apoptotic effect in cardiomyoblasts with hypoxia-induced damage.

TMP downregulated hypoxia-related proteins in a concentration-dependent manner

We measured the hypoxia-related protein levels after co-treatment with TMP under hypoxic conditions. The results indicated that the hypoxia-related proteins HIF-1α, BNIP3, and IGFBP3 were induced by hypoxia, but all were suppressed in a concentration-dependent manner by TMP treatment (Fig. 2A). To confirm whether hypoxia-induced apoptosis acted through HIF-1α to enhance the expression of the BNIP3 and IGFBP3 proteins, we found that BNIP3 and IGFBP3 were significantly downregulated when HIF-1α was blocked by an inhibitor. After BNIP3 and IGFBP3 siRNA transfection, we further observed that the protein
level of activated caspase-3 was decreased with knockdown of BNIP3. In contrast, silencing IGFBP3 could enhance the IGF1 survival pathway, including the p-IGF1 receptor, downstream p-PI3K and p-Akt protein levels (Fig. 2B-D). These data suggest that TMP could have an anti-apoptotic effect through decreased hypoxia-related HIF-1α, BNIP3, and IGFBP3 protein levels induced by hypoxic conditions in H9c2 cardiomyoblast cells.

**TMP completely reversed the apoptotic detrimental effects induced by hypoxia mediated by survival–related p-Akt protein activation**

We further confirmed whether Akt is involved with TMP-mediated cardio-protective mechanisms. The small interfering RNA (siRNA)-mediated specific knockdown of PI3K (class I) was applied to inhibit the activation of downstream Akt. Scramble siRNA, a non-functional and non-targeting siRNA, was used as the control group [35]. The results showed that TMP significantly suppressed PI3K (class I) and blocked downstream Akt activation. Moreover, PI3K siRNA blocked the anti-apoptotic properties of TMP under hypoxia, promoting the activation of caspase-3 (Fig. 3A). After confirmation by flow cytometry, a greater apoptosis ratio was shown in cells receiving TMP plus si-PI3K transfection (10 nM) compared with cells treated with TMP (100 μM) alone (Fig. 3B), indicating that PI3K siRNA blocked the cardio-protective properties of TMP by promoting the activation of caspase-3 in H9c2 myocardial cells.
Hypoxia-induced myocardial cell apoptosis via JNK and p38 MAPK activation was blocked by TMP

To further clarify the cardio-protective effects of TMP in inhibiting hypoxia-induced apoptosis through the MAPK pathway in H9c2 cardiomyoblast cells, we first found using a Western blot assay that TMP reduced the activation of p-JNK, p-ERK, and p-p38 that were enhanced by hypoxia (Fig. 4A). Moreover, p38 MAPK (SB203580) and JNK1/2 (SP600125) inhibitors, but not ERK1/2 (U0126), had suppressive effects on the hypoxia-induced expression of cytochrome c and the active form of caspase-3 in myocardial cells (Fig. 4B), and p38 and JNK siRNA had similar effects (Fig. 4C). Our results suggest that p38 and JNK1/2 MAPK activities are essential for hypoxia-induced cell apoptosis and that TMP exerts its anti-apoptotic effect by inhibiting JNK and the p38 MAPK pathway.

TMP fully rescued hypoxia-induced cell apoptosis of neonatal primary ventricle cardiomyocytes

We further confirmed the functional role of TMP in rat neonatal cardiomyocytes exposed to hypoxic conditions. We treated cells with various concentrations of TMP (10, 50, 100 μM) in a hypoxic environment (<1% O₂), as shown in Fig. 5A. Primary cells were assessed using a TUNEL assay, and we observed that the apoptotic bodies induced by hypoxia were significantly inhibited by TMP treatment in a concentration-dependent manner. Ventricular cardiomyocytes were harvested after treatment. The levels of the survival proteins p-Akt and Bcl-2, as well as the pro-apoptotic Bcl-2 family members Bax and BNIP3, were analyzed by immunoblotting. The survival-related proteins p-Akt and Bcl-2 were significantly recovered. Apoptotic Bax and BNIP3 levels were reduced in the presence of TMP, compared with cells only exposed to hypoxia (Fig. 5B). Based on these findings, we strongly suggest that TMP protects against the pro-apoptotic effects induced by hypoxia in cardiomyocytes.
Discussion

This study demonstrated that a hypoxic environment directly induces myocardial cell apoptosis via HIF-1α, p38 MAPK and JNK to upregulate the hypoxia-related proteins BNIP3 and IGFBP3. Administration of TMP significantly inhibited hypoxia-induced cell death, as
demonstrated by TUNEL and flow cytometry (Fig. 1). Furthermore, the suppression of cell damage by TMP was confirmed by neonatal primary culture. Our data suggest that TMP could inhibit the expression of BNIP3 and provide cardio-protective properties by dramatically suppressing the active forms of caspase-3 and cytochrome c released by myocardial cells. We also found that TMP blocked the effect of IGFBP3 to enhance cell survival by upregulating the activities of the IGF-1 receptor and p-Akt in hypoxia-exposed myocardial cells (Fig. 2). Additional evidence has shown that TMP has an anti-inflammatory effect via MAPK pathway suppression [34]. Some authors have suggested that TMP may be beneficial in the treatment of cardiovascular disorders such as atherosclerosis through MAPK inhibition [33]. We found
that hypoxia-mediated heart injury was not only mediated by the HIF-1α transcription factor but also through p38 and JNK1/2 MAPK activity, as determined by inhibitors (SB258003 and SP600125) and siRNA, resulting in the marked inhibition of hypoxia-induced cell apoptosis. We also found that hypoxia-treated myocardial cells underwent apoptosis through JNK1/2 and p38 MAPK activation, but not Erk1/2, which suggests that JNK1/2 or p38 MAPK may also be key mediators of the downstream signals under hypoxic injury, which may suppress the PI3K/Akt pathway [36]. Many studies have indicated that the activation of the PI3K/Akt pathway is involved in the anti-apoptotic effects of stimuli and that it plays a central role in cellular survival in many different types of cells [21]. As we blocked Akt using a PI3K inhibitor (LY294002) and PI3K siRNA, the TMP-mediated cardio-protective properties under hypoxia exposure were completely blocked. Our results imply that TMP-mediated cardio protection via the activated IGF-1R survival pathway and Akt activation was truly required for TMP to rescue the hypoxia-mediated injuries. Moreover, neonatal primary culture confirmed our H9c2 cardiomyoblast cell result (Fig. 5).

Apoptosis has been shown to play an important role in heart failure pathogenesis in various etiologies, such as chronic pressure overload or acute myocardial infarction [9]. The Bcl-2 family is important in balancing the mitochondrial potential between the up- and downregulation of the pro-apoptotic (Bax, Bad) and anti-apoptotic (Bcl-xL, Bcl-2) protein members, which determines the fate of the cells to either undergo apoptosis or survive. This study further determined that TMP could inhibit cell death by downregulating the pro-apoptotic Bax and caspase-3 proteins, which are enhanced by hypoxia. TMP upregulated the Bcl-2 protein level to maintain the mitochondrial membrane potential and inhibited cytochrome c release in H9c2 myocardial cells. Our findings suggest that TMP, which is the most important active compound in Chuan Xiong, could maintain the mitochondrial membrane potential and further provide a strong protective effect in ischemic heart injury. As we know, Chuan Xiong is widely used in Chinese herbal medicine in the treatment of ischemic cerebral vascular diseases and coronary arteries [25]. TMP may therefore be a potential drug candidate to restore hypoxic/ischemic heart disease.

In summary, dilated cardiomyopathy (DCM) and coronary artery disease (CAD) will eventually lead to heart failure. Both of these diseases would lead to myocardial hypoxic/ischemic conditions. Trauma can also induce hemorrhagic shock, resulting in the significant loss of intravascular volume and leading sequentially to hemodynamic instability, decreased tissue perfusion, cellular hypoxia, organ damage and death. This type of ischemic situation is due to the interruption of the oxygen and blood supply. A large amount of evidence has shown that hypoxia, oxidative stress and ischemia/reperfusion damage result in cardiac pathologies, such as heart failure, hypertensive cardiac hypertrophy, cardiomyopathies and cardiomyocyte apoptosis [37]. Studies have also shown that the incidence of sepsis or multiple organ dysfunction cause death after heart failure. Evidence suggests that under hypoxic conditions, dimer formation of the stabilized HIF-1α with the HIF-1β subunit stimulate transcriptional activity of a wide range of genes, including those involved in cell metabolism, hypoxia tolerance and cell apoptosis. The role of HIF-1α and its related genes in cardiac ischemic injury remains incompletely understood (Fig. 6). Our study demonstrated that HIF-1α activates downstream IGFBP3 to block the IGF-1R/PI3K/Akt pathway and promote abundant BNIP3 expression, which could enhance cytochrome c release and cause myocardial cell death. However, TMP efficiently rescues hypoxia-induced myocardial cell damage (Fig. 6). Our findings strongly suggest that this component of Chuan Xiong could be important to our health, decreasing the rate of mortality and heart dysfunction in acute myocardial infarction or hemorrhagic shock.

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Disclosure Statement

The authors have nothing to disclose.

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