Tetramethylpyrazine reverses high-glucose induced hypoxic effects by negatively regulating HIF-1α induced BNIP3 expression to ameliorate H9c2 cardiomyoblast apoptosis

Shih-Ping Liu[1], Marthandam Asokan Shibu[2], Fuu-Jen Tsai[3,4], Yuan-Man Hsu[5], Chang-Hai Tsai[4], Jing-Gung Chung[5], Jai-Sing Yang[6], Chih-Hsin Tang[7], Shulin Wang[8], Qiaowen Li[8†] and Chih-Yang Huang[2,7,9*†]

Abstract

Background: Diabetic patients are highly vulnerable to hypoxic injury, which is associated with hypoxia induced BNIP3 expression that subsequently activate apoptosis. Our previous research show that Tetramethylpyrazine (TMP), a food flavoring agent, represses the hypoxia induced BNIP3 expression attenuate myocardial apoptosis. In this study, we evaluate the effect of TMP to provide protection against hypoxia aggravated high-glucose associated cellular apoptosis.

Methods: The cytoprotective effect of TMP against high glucose induced cellular damages was determined on embryo derived H9c2 cardiomyoblast cells that were subjected to 5% hypoxia for 24 h and subjected to different duration of 33 mM high glucose challenge. Further, the involvement of HIF-1α and BNIP3 in cellular damage and the mechanism of protection of TMP were determined by overexpression and silencing HIF-1α and BNIP3 protein expression.

Results: The results show that hypoxic effects on cell viability aggravates with high glucose challenge and this augmentative effect is mediated through BNIP3 in H9c2 cardiomyoblast cells. However, TMP administration effectively reversed the augmented HIF-1α levels and BNIP3 elevation. TMP improved the survival of H9c2 cells and effectively suppressed apoptosis in H9c2 cells. Further comparison on the effects of TMP on H9c2 cells challenged with high glucose and those challenged with hypoxia show that TMP precisely regulated the hypoxic intensified apoptotic effects in high-glucose condition.

Conclusion: The results clearly show that flavoring agent-TMP attenuates cytotoxicity amplified by hypoxia challenge in high glucose condition by destabilizing HIF-1α.

Keywords: HIF-1, Diabetes mellitus, Food flavoring, Caspase, Hypoxia

* Correspondence: cyhuang@mail.cmu.edu.tw
†Qiaowen Li and Chih-Yang Huang contributed equally to this work.
²College of Medicine, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Tzu Chi University, Hualien, Taiwan
⁷Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan
Full list of author information is available at the end of the article

Background

Coronary heart disease is a critical condition that accounts for about 80% of death among people with diabetes. Diabetic patients are more prone to cardiac events such as stroke as diabetes mellitus is associated with macrovascular manifestations such as atherosclerosis and medial calcification [1, 2]. Numerous studies have revealed hyperglycemia as an independent risk factor that can directly inflict cardiac damage, thereby contributing to diabetic cardiomyopathy [3]. Initial responses of cardiac cells to hyperglycemic condition include abnormal metabolism, abnormal fetal gene expression and defects in cellular organelles [4]. Cardiomyocyte death follows as a consequence to these abnormalities and forms a reason for subsequent collagen accumulation and reduced contractile function [5].

Although, diabetes is an independent risk factor for CVD, evidences show that hyperglycemia, contributes to myocardial damage following ischemic events [6].

Hypoxic conditions are seen to be the general cause of cardiac cell death in pathological conditions including diabetes [7–9]. Previous studies have shown that the probable mechanism of hypoxia-induced apoptosis is through hypoxia induced stabilization and nuclear translocation of hypoxia-inducible factor-1α (HIF-1α) protein. Activation of HIF-1α induces downstream proteins including Bcl-2 adenovirus E1B nineteen-kilodalton interacting protein 3 (BNIP3) and insulin-like growth factor binding protein 3 (IGFBP-3) expression. BNIP3 forms a stable homodimeration complex that is inserted into the mitochondrial membrane causing mitochondrial damage resulting in mitochondria-dependent apoptosis. In addition, IGFBP-3 enhances cellular apoptosis by sequestering the survival factor IGF-1 thereby blocks the IGF1R/PI3K/Akt signaling activation [10–12]. Such events associated with excessive hypoxia have a strong correlation with cardiac diseases.

Under normoxia conditions, the transcription of the *bnip3* gene is repressed by transcription factors NFκB and E2F4 [13]. In addition, *bnip3* is one of the most abundant genes induced by HIF-1 under hypoxic conditions. BNIP3 mRNA and protein levels are enhanced due to a HRE that occurs in the proximal promoter for *bnip3* that binds to the heterodimer of HIF-1, inducing BNIP3 expression [14]. Hypoxia-induced high-level transcription of *bnip3* is mediated by E2F1 releasing from E2F4 and FoxO3 to enhance HIF-1α transcriptional function [15–17]. Our previous studies show that prolonged-hypoxia induced HIF-1α stimulated BNIP3 and enhanced IGFBP-3 activation and their effect on survival pathway and mitochondria-dependent cardiomyocyte apoptosis are similar in neonatal cardiomyocytes and in H9c2 cardiomyoblast cells [18]. H9c2 cell lines show a identical hypertrophic responses as those observed in primary neonatal cardiomyocytes when exposed to hypertrophic factors like angiotensin-II, endothelin-1 [19]. The cell line is also used for cardiotoxicity analyses and to understand stress associated mechanisms and myocyte damage including apoptosis and necrosis [20]. Moreover, H9c2 cells are well known to mimic primary cardiomyocytes in their responses to hypoxia and in addition, they are more similar to primary cardiomyocytes in their energy metabolism patterns. Therefore, H9c2 cells are more appropriate to simulate cardiac ischemic effects [21].

Previous reports show that, Tetramethylpyrazine (TMP) found in food products such as potato fries, fermented cocoa bean, tea, coffee, bread, beer, spirits, peanuts and various dairy-foods is a potential food ingredient to provide cardio-protection. Our previous reports elucidated the rescue effect of TMP on H9c2 cells against hypoxia-induced apoptosis that involves suppression of HIF-1α, BNIP3 and IGFBP3 [22–24]. In the present study, we further explore the beneficial effects of TMP induced suppression BNIP3 on hypoxia aggravated cardiac apoptosis in hyperglycemic condition.

Materials and methods

Cell culture and treatment

H9c2 cardiomyoblasts from the American Type Culture Collection (ATCC,CRL-1446) (Rockville, MD, USA) were cultured in 100-mm or 60-mm culture dishes in Dulbecco's modified Eagles medium (DMEM, Sigma-Aldrich, USA) supplemented with 100 μg/mL penicillin (Gibco, Waltham, MA, USA), 100 μg/mL streptomycin (Gibco), 2 mM glutamine (Gibco), and 10% Clontech fetal bovine serum (Hyclone, GE Healthcare Life Sciences, Pittsburgh, PA, USA) inside humidified air (5% CO_2) at 37 °C. The control cells were exposed to media containing 5.5 mM D-glucose and for high-glucose challenge the cells were exposed to 33 mM D-glucose according to previous reports [4, 7, 25].

Examination of protein expressions by Western blotting

A total of 5×10^5 cells of H9c2 cells were plated onto 10 cm dish and incubated at 37 °C for treated with high glucose for 12 h and then combined in the hypoxia environment and for another 24 h. To isolate total proteins, 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 (Bio-rad, USA). Samples containing equal amounts of protein (50 μg) were loaded and analyzed using Western blot analysis. Briefly, proteins were separated by 10% SDS-

PAGE and transferred onto PVDF membrane (Millipore, Belford, Massachusetts, USA). 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) (Sigma-Aldrich, St. Louis, MO, USA) for at least 1 h at room temperature. 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), BNIP3, cleaved caspase-9, cleaved caspase-3, phosphorylated-Akt Ser473 (p-Akt) (Cell Signaling, Danvers, MA, USA), in the above solution on an orbit shaker at 4 °C overnight. Following primary antibody incubations, membranes were incubated with horseradish peroxidase-linked secondary antibodies (anti-rabbit, anti-mouse, or anti-goat IgG) (Santa Cruz).

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). H9c2 were stained 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 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.

siRNA transfection

Double-stranded siRNA sequences targeting HIF-1α and BNIP3 mRNAs were obtained from Sigma-Aldrich (St. Louis, Missouri, USA). The non-specific siRNA (scramble) was non-targeting. Cells were cultured in 60-mm well plates in medium. siRNA transfection was carried out with transfection reagent (PureFection™, System Biosciences, Mountain View, CA.) following the manufacturer's protocol. Specific silencing was confirmed by immune blotting with cellular extracts after transfection.

Gene overexpression through transient transfection

Cells with 50% confluence were placed into fresh culture medium containing serum 2 h before the transient transfection. pCDNA3-HAyun-FoxO3a [26] plasmids were then transfected into the cells for 24 h using the PureFection™ Nanotechnology-based Transfection Reagent (System Biosciences, CA, USA) following the manufacturer's protocol. In each experiment, the gene overexpression efficiency was measured by western blot (data not shown).

Results

High glucose aggravates hypoxia induced BNIP3 gene expression in H9c2 cardiomyoblast cells

H9c2 cells under 33 mM high glucose when exposed to hypoxic conditions for 24 h showed aggravated increase in BNIP3 expression in a time dependent manner (Fig. 1). Increase in BNIP3 expression was also correlated with increase in pro-apoptotic proteins such as Bak and decrease in cell survival associated anti-apoptotic proteins such as p-Akt and BcL-xL.

High glucose stress under hypoxia enhances HIF-1α nuclear translocation and induces BNIP3 expression

To ensure whether high glucose can aggravate hypoxia induced cardiomyoblast apoptosis we exposed H9c2 cells to high glucose conditions and hypoxia and analyzed by flow cytometry. The results show that the synergistic effect of hypoxia and high glucose stress elevated the apoptotic cell death (Fig. 2a) which also correlation with BNIP3 levels (Fig. 2b). Further, hypoxia induced-Bnip3 was significant inhibited when BNIP3 was silenced by siRNA transfection and the reduction in BNIP3 levels were correlated with reduction in apoptosis (Fig. 2).

TMP inhibits cellular apoptosis triggered by high glucose stress under hypoxia and downregulates BNIP3 levels

To investigate the effect of TMP on H9c2 cells under synergistic hypoxia and high glucose stress 5 μM, 10 μM and 20 μM of TMP was treated to the cells cultured in high glucose media under hypoxia. The Western blotting results show that treatment with TMP dose-dependently suppressed the levels of HIF-1α and BNIP3. Reduction in the levels of HIF-1α and BNIP3 was also correlated with increase in the level of p-Akt and BcL-xl (Fig. 3a). Flow cytometric analysis also show that treatment with TMP reduces in the cellular apoptosis induced by the synergistic effect of high glucose and hypoxia (Fig. 3b). The TUNEL assay also shows that TMP reduces the number of apoptotic nuclei in H9c2 cell challenged with both hypoxia and high glucose (Fig. 3c).

TMP attenuates the catastrophic apoptotic events induced by high glucose under hypoxia regulating the synergistic increase BNIP3 expression in H9c2 cells

We further over expressed HIF-1α and BNIP3, incubated with high glucose and hypoxia, and treat with 10 μM of TMP. We found that both HIF-1α and BNIP3 increased during hypoxia but the expression of apoptotic markers were considerably increased when co-challenged with both high glucose and hypoxia. Further increase in BNIP3 and HIF-1α by overexpression also showed elevated caspase activation. However, treatment with TMP was more effective in regulating BNIP3 associated caspase activation (Fig. 4).
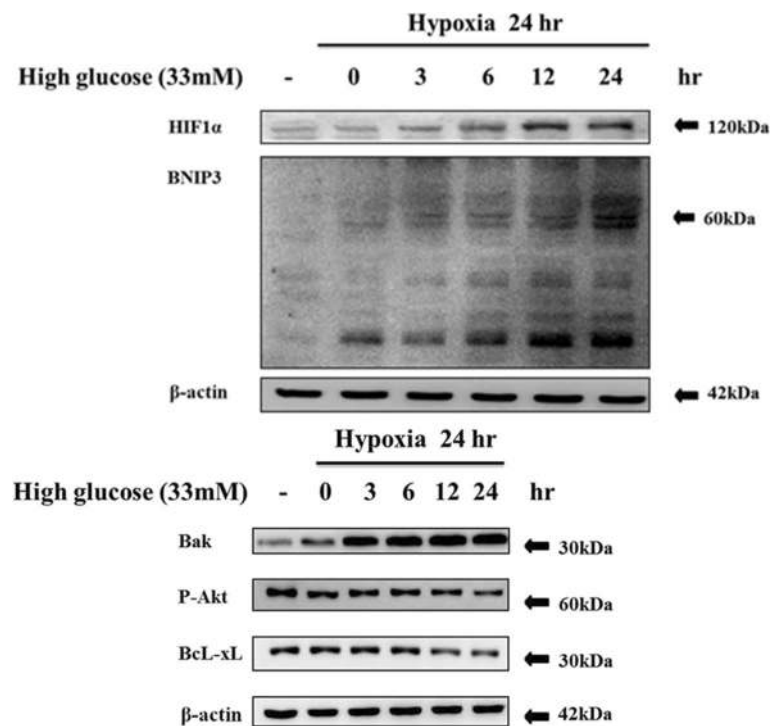
Liu *et al. Nutrition & Metabolism*   (2020) 17:12

Page 4 of 9

Fig. 1 Effects of high-glucose and hypoxia on BNIP3 expression in H9c2 cells. Cells were cultured in high-glucose medium (33 mM) and incubated in hypoxia (5%) for different time-periods as indicated. Wetern blotting analysis show increase in hypoxia associated protein expression such as HIF1α and BNIP3 that was correlated with increase in pro-apoptotic protein Bak and decrease in anti-apoptotic proteins P-Akt and BcL-xLl($n = 3$).

In summary, our results show that hypoxia exposure under high-glucose challenge triggers aggravated cellular apoptosis via elevation of BNIP3 levels and TMP administration effectively inhibited high-glucose aggravated hypoxic cell death via down regulation of BNIP3 expression.
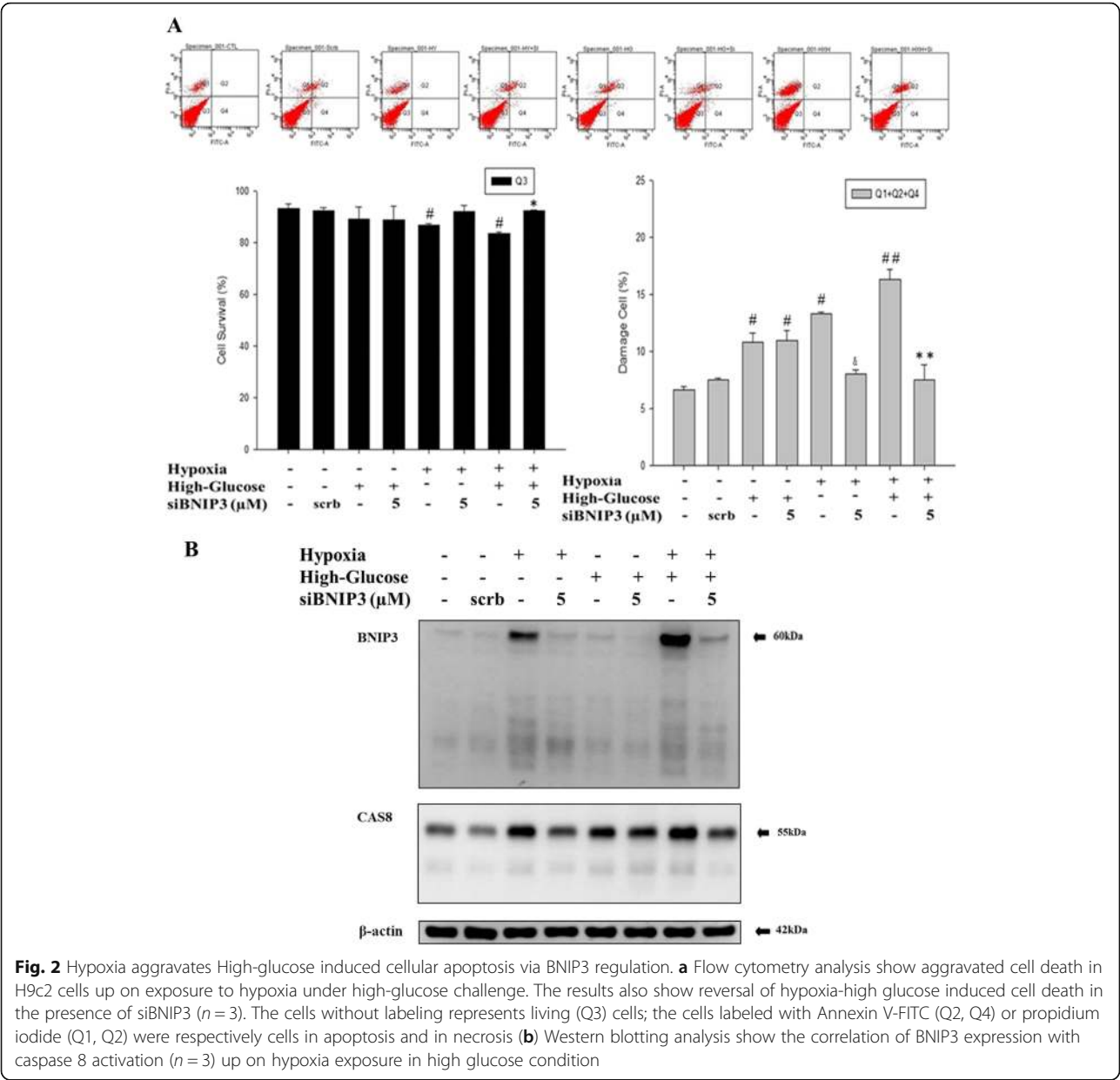
Discussion

Chronic hypoxia in the presence of high glucose leads to progressive acidosis of cardiomyocytes in culture. We previously demonstrated HIF-1α as an important transcription factor in ischemic embryo rat heart [27, 28] [29], and increased protein level of HIF-1α is associated with high glucose treatment. Another study indicated that hypoxia/acidosis genes such as BNIP3, IGFBP-3, hemeoxygenase 1 (HO-1), pyruvate dehydrogenase kinase 1 (PDK1), prolyl hydroxylases (PHDs) and HIF-2 were upregulated during ischemia [30]. This study evaluates the involvement of HIF-1α and BNIP3 in hypoxic condition under high glucose stress.

A critical response to hypoxia in cells and tissues is mediated through changes in the transcriptional induction of a series of genes that participate in angiogenesis, iron metabolism, glucose metabolism, and cell proliferation/survival that are regulated by the hypoxia-inducible factors (HIFs) [31, 32]. In this study, we found that

hypoxia induced BNIP3 expression is enhanced by high glucose in a time dependent manner in H9c2 cardiomyoblast cells. Further, HIF-1α expression was found to be directly proportional to BNIP3 levels in hypoxic condition which was further elevated in high-glucose condition. Moreover, apoptosis in H9c2 cardiomyoblast cells also increased with increasing BNIP3 levels observed in hypoxic condition.

Chronic hyperglycemia is the major cause to many diabetic complications. Generally, cells regulate the glucose transport during hyperglycemia in order to maintain cellular metabolic homeostasis. Disturbances in the glucose homeostasis in diabetic condition is associated with deterioration in the glucose transport. BNIP3 is a BH3 protein that promotes mitochondrial destruction and triggers mitochondrial death by increasing the mitochondrial membrane permeability and subsequent release of cytochrome *c*. Our data suggest that BNIP3 expression is significantly upregulated following 24 h of hypoxic stress in H9C2 cardiomyoblast under high glucose condition.

Previous reports showed that HIF-1α induced by hypoxia-acidosis could target the promoter of BNIP3 to transcribe these genes and further induce programmed cell death. Here, we further evaluated if elevated HIF-1α mediate BNIP3 to enhance cardiomyocyte apoptosis.

Fig. 2 Hypoxia aggravates High-glucose induced cellular apoptosis via BNIP3 regulation. **a** Flow cytometry analysis show aggravated cell death in H9c2 cells up on exposure to hypoxia under high-glucose challenge. The results also show reversal of hypoxia-high glucose induced cell death in the presence of siBNIP3 ($n = 3$). The cells without labeling represents living (Q3) cells; the cells labeled with Annexin V-FITC (Q2, Q4) or propidium iodide (Q1, Q2) were respectively cells in apoptosis and in necrosis (**b**) Western blotting analysis show the correlation of BNIP3 expression with caspase 8 activation ($n = 3$) up on hypoxia exposure in high glucose condition

BNIP3 has been reported to be a potent inducer of autophagy in adult rat cardiac myocytes. The present results show that BNIP3 expression is augmented by high glucose under hypoxic condition and resulted in mitochondria-dependent apoptosis in cardiomyocytes. These findings clearly indicate that HIF-1α-induced BNIP3 signaling is an important modulator of hypoxia-induced mitochondrial apoptosis. A previous study showed that BNIP3 activity is induced by hypoxia in an HIF1-dependent manner [33–36].

Our previous results show that food ingredients and phytochemicals exhibit various diverse biological activities [37–41]. Particularly various phytoextracts

and their active principles have shown to provide effective cardioprotection against various challenges [42–50]. Cocoa consumption has been previously linked to cardiac health and various mechanisms have been proposed for the cardioprotective effects of cocoa [51]. TMP is one of the major bioactive component of cocoa liquors and in Chinese black vinegar [52, 53]. Moreover, *Ligusticum chuanxiong Hort*, a commonly used traditional Chinese medicine used for empiric treatment of cerebrovascular and cardiovascular diseases for many centuries also contains TMP as its major bioactive principle. This traditional Chinese medicines has been widely used
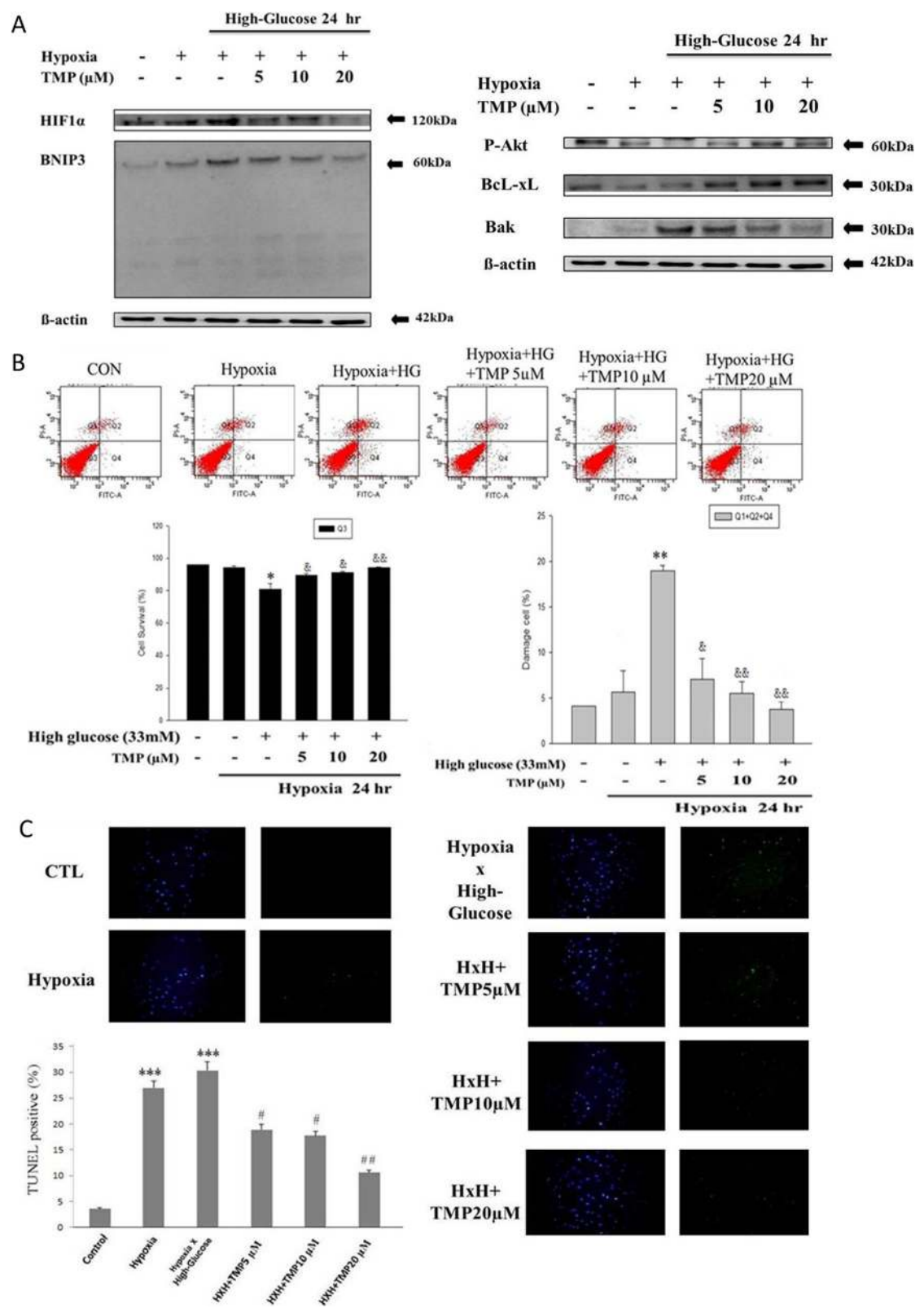
Liu *et al. Nutrition & Metabolism* (2020) 17:12

Page 6 of 9

Fig. 3 (See legend on next page.)

(See figure on previous page.)

Fig. 3 Effect of Tetramethylpyrazine on elevated HIF-1α and the downstream BNIP3 in H9c2 cells under hypoxia and high glucose condition. **a** Western blotting analysis (*n* = 3) show increase in both HIF1α and BNIP3 levels under hypoxia in high-glucose conditions. **b** Flow cytometry analysis (*n* = 3) show TMP induced modulation on the effect of hypoxia in H9c2 cells under high glucose condition; cells without labeling represents living (Q3) cells; the cells labeled with Annexin V-FITC (Q2, Q4) or propidium iodide (Q1, Q2) were respectively cells in apoptosis and in necrosis. **c** Microphotographs (400X magnification) of TUNEL assay show the ameliorating effect of TMP on cell death induced by hypoxia under high-glucose condition (*n* = 3)

in clinical treatments for vascular disease, and it has been efficient in animal stroke models and is known to preserve the structural and functional integrity of mitochondria [22]. Our previous study has shown that *Ligusticum chuanxiong* extracts inhibits the inflammatory markers p-NFkB and increases the survival marker p-Akt expression. TMP, also known as ligustrazine is known to possess a wide range of biological activities including antioxidant, anti-inflammatory, antitumor, properties and provide cyto-protection to kidneys, brain and heart [23, 54–57]. TMP is also known to improve microcirculation and can be potentially used to treat thrombosis as they attenuate platelet aggregation [58, 59].

Our results reveal that TMP inhibits cell apoptosis of H9c2 cells in high-glucose condition with hypoxia, a condition that imitate those in diabetes patients during ischemic injury. We further observed that HIF-1α and BNIP3 induced cell apoptosis in H9C2 cardiomyoblast cells increases during hypoxia and is further fortified in hypoxia with high-glucose stress, however the effects of BNIP3 was significantly inhibited by TMP treatment.

Fig. 4 TMP attenuates hypoxia and high-glucose induced BNIP3 expression and apoptosis. Western blotting analysis (*n* = 3) show TMP induced modulation in HIF1α and BNIP3 levels in H9c2 cells under hypoxia and in hypoxia in combination with high-glucose and in cell overexpressing either HIF1α or BNIP3

Conclusions

In this study, by using over expression of HIF-1α and BNIP3 and with TMP treatment, we concluded that high glucose associated hypoxia induced myocardial cell apoptosis mediated by HIF-1α dependent signaling and the activation of BNIP3 is attenuated with TMP treatment.

Availability of data and materials
The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Author details
[1]Graduate Institute of Biomedical Sciences, China Medical University, Taichung, Taiwan. [2]College of Medicine, Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation, Tzu Chi University, Hualien, Taiwan. [3]School of Chinese Medicine, College of Chinese Medicine, China Medical University, Taichung 40402, Taiwan. [4]China Medical University Children's Hospital, China Medical University, Taichung, Taiwan. [5]Department of Biological Science and Technology, China Medical University, Taichung, Taiwan. [6]Department of Pharmacology, School of Medicine, China Medical University, Taichung, Taiwan. [7]Department of Medical Research, China Medical University Hospital, China Medical University, Taichung, Taiwan. [8]Department of Cardiology, The Sixth Affiliated Hospital of Guangzhou Medical University, Qingyuan People's Hospital, Qingyuan 511518, Guangdong, China. [9]Department of Biotechnology, Asia University, Taichung, Taiwan.
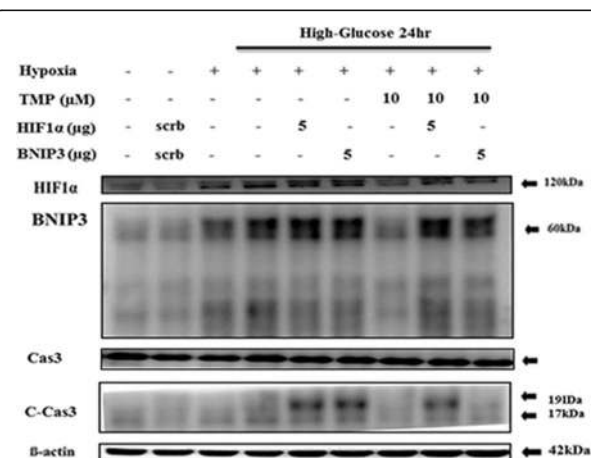
Liu *et al. Nutrition & Metabolism* (2020) 17:12

Page 8 of 9

References

1. Creager MA, Lüscher TF, Cosentino F, Beckman JA. Diabetes and vascular disease. Circulation. 2003;108:1527–32.
2. Thomas RJ, Palumbo PJ, Melton LJ 3rd, Roger VL, Ransom J, O'Brien PC, Leibson CL. Trends in the mortality burden associated with diabetes mellitus: a population-based study in Rochester, Minn, 1970-1994. Arch Intern Med. 2003;163:445–51.
3. Kenny HC, Abel ED. Heart failure in type 2 diabetes mellitus. Circ Res. 2019; 124:121–41.
4. Cai L, Li W, Wang G, Guo L, Jiang Y, Kang YJ. Hyperglycemia-Induced Apoptosis in Mouse Myocardium. Mitochondrial Cytochrome c–Mediated Caspase-3 Activation Pathway . Diabetes. 2002;51:1938–48.
5. Feuerstein GZ, Young PR. Apoptosis in cardiac diseases: stress- and mitogen-activated signaling pathways. Cardiovasc Res. 2000;45:560–9.
6. Dokken BB. The pathophysiology of cardiovascular disease and diabetes: beyond blood pressure and lipids. Diabetes Spectrum. 2008;21:160–5.
7. Hu M, Ye P, Liao H, Chen M, Yang F. Metformin protects H9C2 Cardiomyocytes from high-glucose and hypoxia/Reoxygenation injury via inhibition of reactive oxygen species generation and inflammatory responses: role of AMPK and JNK. J Diab Res. 2016;2016:9.
8. Zhang Y, Su W, Zhang Q, Xu J, Liu H, Luo J, Zhan L, Xia Z, Lei S. Glycine Protects H9C2 Cardiomyocytes from High Glucose- and Hypoxia/Reoxygenation-Induced Injury via Inhibiting PKCβ2 Activation and Improving Mitochondrial Quality. J Diab Res. 2018;2018:8.
9. Sada K, Nishikawa T, Kukidome D, Yoshinaga T, Kajihara N, Sonoda K, Senokuchi T, Motoshima H, Matsumura T, Araki E. Hyperglycemia induces cellular hypoxia through production of mitochondrial ROS followed by suppression of Aquaporin-1. PLoS One. 2016;11:e0158619.
10. Graham RM, Frazier DP, Thompson JW, Haliko S, Li H, Wasserlauf BJ, Spiga MG, Bishopric NH, Webster KA. A unique pathway of cardiac myocyte death caused by hypoxia-acidosis. J Exp Biol. 2004;207:3189–200.
11. Harris AL. Hypoxia--a key regulatory factor in tumour growth. Nat Rev Cancer. 2002;2:38–47.
12. Kim JY, Ahn HJ, Ryu JH, Suk K, Park JH. BH3-only protein Noxa is a mediator of hypoxic cell death induced by hypoxia-inducible factor 1alpha. J Exp Med. 2004;199:113–24.
13. Shaw J, Kirshenbaum LA. Molecular regulation of autophagy and apoptosis during ischemic and non-ischemic cardiomyopathy. Autophagy. 2008;4:427–34.
14. Kothari S, Cizeau J, McMillan-Ward E, Israels SJ, Bailes M, Ens K, Kirshenbaum LA, Gibson SB. BNIP3 plays a role in hypoxic cell death in human epithelial cells that is inhibited by growth factors EGF and IGF. Oncogene. 2003;22:4734–44.
15. Chinnadurai G, Vijayalingam S, Gibson SB. BNIP3 subfamily BH3-only proteins: mitochondrial stress sensors in normal and pathological functions. Oncogene. 2008;27(Suppl 1):S114–27.
16. Mammucari C, Schiaffino S, Sandri M. Downstream of Akt: FoxO3 and mTOR in the regulation of autophagy in skeletal muscle. Autophagy. 2008;4:524–6.
17. Yurkova N, Shaw J, Blackie K, Weidman D, Jayas R, Flynn B, Kirshenbaum LA. The cell cycle factor E2F-1 activates Bnip3 and the intrinsic death pathway in ventricular myocytes. Circ Res. 2008;102:472–9.
18. Feng CC, Lin CC, Lai YP, Chen TS, Marthandam Asokan S, Lin JY, Lin KH, Viswanadha VP, Kuo WW, Huang CY. Hypoxia suppresses myocardial survival pathway through HIF-1alpha-IGFBP-3-dependent signaling and enhances cardiomyocyte autophagic and apoptotic effects mainly via FoxO3a-induced BNIP3 expression. Growth Factors. 2016;34:73–86.
19. Watkins SJ, Borthwick GM, Arthur HM. The H9C2 cell line and primary neonatal cardiomyocyte cells show similar hypertrophic responses in vitro. In Vitro Cell Dev Biol Anim. 2011;47:125–31.
20. Witek P, Korga A, Burdan F, Ostrowska M, Nosowska B, Iwan M, Dudka J. The effect of a number of H9C2 rat cardiomyocytes passage on repeatability of cytotoxicity study results. Cytotechnology. 2016;68:2407–15.
21. Kuznetsov AV, Javadov S, Sickinger S, Frotschnig S, Grimm M. H9c2 and HL-1 cells demonstrate distinct features of energy metabolism, mitochondrial function and sensitivity to hypoxia-reoxygenation. Biochim Biophys Acta. 1853;2015:276–84.
22. Or TC, Yang CL, Law AH, Li JC, Lau AS. Isolation and identification of anti-inflammatory constituents from Ligusticum chuanxiong and their underlying mechanisms of action on microglia. Neuropharmacology. 2011;60:823–31.
23. Fan L, Wang K, Shi Z, Die J, Wang C, Dang X. Tetramethylpyrazine protects spinal cord and reduces inflammation in a rat model of spinal cord ischemia-reperfusion injury. J Vasc Surg. 2011;54:192–200.
24. Lin K-H, Kuo W-W, Jiang A-Z, Pai P, Lin J-Y, Chen W-K, Day C, Shen C-Y, Padma V, Huang C. Tetramethylpyrazine ameliorated hypoxia-induced myocardial cell apoptosis via HIF-1?/JNK/p38 and IGFBP3/BNIP3 inhibition to upregulate PI3K/Akt survival signaling. Cell Physiol Biochem. 2015;36:334–44.
25. Feng C-C, Pandey S, Lin C-Y, Shen C-Y, Chang R-L, Chang T-T, Chen R-J, Viswanadha VP, Lin Y-M, Huang C-Y. Cardiac apoptosis induced under high glucose condition involves activation of IGF2R signaling in H9c2 cardiomyoblasts and streptozotocin-induced diabetic rat hearts. Biomed Pharmacother. 2018;97:880–5.
26. Feng C-C, Lin C-C, Lai Y-P, Chen T-S, Marthandam Asokan S, Lin J-Y, Lin K-H, Viswanadha VP, Kuo W-W, Huang C-Y. Hypoxia suppresses myocardial survival pathway through HIF-1α-IGFBP-3-dependent signaling and enhances cardiomyocyte autophagic and apoptotic effects mainly via FoxO3a-induced BNIP3 expression. Growth Factors. 2016;34:73–86.
27. Lee SH, Wolf PL, Escudero R, Deutsch R, Jamieson SW, Thistlethwaite PA. Early expression of angiogenesis factors in acute myocardial ischemia and infarction. N Engl J Med. 2000;342:626–33.
28. Pfeffer MA, Braunwald E. Ventricular remodeling after myocardial infarction. Experimental observations and clinical implications. Circulation. 1990;81:1161–72.
29. Hwang JM, Weng YJ, Lin JA, Bau DT, Ko FY, Tsai FJ, Tsai CH, Wu CH, Lin PC, Huang CY, Kuo WW. Hypoxia-induced compensatory effect as related to Shh and HIF-1alpha in ischemia embryo rat heart. Mol Cell Biochem. 2008;311:179–87.
30. Pitts KR, Derry JM, Kerkof K, Lawrence WA, Toombs CF. Differentially regulated functional gene clusters identified during ischemia and reperfusion in isolated cardiac myocytes using coverslip hypoxia. J Pharmacol Toxicol Methods. 2008;57:42–51.
31. Giaccia AJ, Simon MC, Johnson R. The biology of hypoxia: the role of oxygen sensing in development, normal function, and disease. Genes Dev. 2004;18:2183–94.
32. Ke Q, Costa M. Hypoxia-inducible factor-1 (HIF-1). Mol Pharmacol. 2006;70:1469–80.
33. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. Nature. 2008;451:1069–75.
34. Scott RC, Juhasz G, Neufeld TP. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. Curr Biol. 2007;17:1–11.
35. Bursch W. The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ. 2001;8:569–81.
36. Hsieh YC, Athar M, Chaudry IH. When apoptosis meets autophagy: deciding cell fate after trauma and sepsis. Trends Mol Med. 2009;15:129–38.
37. Wu KM, Hsu YM, Ying MC, Tsai FJ, Tsai CH, Chung JG, Yang JS, Tang CH, Cheng LY, Su PH, et al. High-density lipoprotein ameliorates palmitic acid-induced lipotoxicity and oxidative dysfunction in H9c2 cardiomyoblast cells via ROS suppression. Nutr Metab (Lond). 2019;16:36.
38. Liu S-C, Tsai C-H, Wu T-Y, Tsai C-H, Tsai F-J, Chung J-G, Huang C-Y, Yang J-S, Hsu Y-M, Yin M-C, et al. Soya-cerebroside reduces IL-1β-induced MMP-1 production in chondrocytes and inhibits cartilage degradation: implications for the treatment of osteoarthritis. Food Agric Immunol. 2019;30:620–32.
39. Lin CC, Chen KB, Tsai CH, Tsai FJ, Huang CY, Tang CH, Yang JS, Hsu YM, Peng SF, Chung JG. Casticin inhibits human prostate cancer DU 145 cell migration and invasion via Ras/Akt/NF-kappaB signaling pathways. J Food Biochem. 2019;43:e12902.
40. Lee H-P, Chen P-C, Wang S-W, Fong Y-C, Tsai C-H, Tsai F-J, Chung J-G, Huang C-Y, Yang J-S, Hsu Y-M, et al. Plumbagin suppresses endothelial progenitor cell-related angiogenesis in vitro and in vivo. J Funct Foods. 2019;52:537–44.
41. Tsai CY, Fang HY, Shibu MA, Lin YM, Chou YC, Chen YH, Day CH, Shen CY, Ban B, Huang CY. Taiwanin C elicits apoptosis in arecoline and 4-nitroquinoline-1-oxide-induced oral squamous cell carcinoma cells and hinders proliferation via epidermal growth factor receptor/PI3K suppression. Environ Toxicol. 2019;34:760–7.
42. Hsieh YL, Shibu MA, Lii CK, Viswanadha VP, Lin YL, Lai CH, Chen YF, Lin KH, Kuo WW, Huang CY. Andrographis paniculata extract attenuates

pathological cardiac hypertrophy and apoptosis in high-fat diet fed mice. J Ethnopharmacol. 2016;192:170–7.

43. Chen PY, Hou CW, Shibu MA, Day CH, Pai P, Liu ZR, Lin TY, Viswanadha VP, Kuo CH, Huang CY. Protective effect of co-enzyme Q10 on doxorubicin-induced cardiomyopathy of rat hearts. Environ Toxicol. 2017;32:679–89.

44. Chen YF, Shibu MA, Fan MJ, Chen MC, Viswanadha VP, Lin YL, Lai CH, Lin KH, Ho TJ, Kuo WW, Huang CY. Purple rice anthocyanin extract protects cardiac function in STZ-induced diabetes rat hearts by inhibiting cardiac hypertrophy and fibrosis. J Nutr Biochem. 2016;31:98–105.

45. Tsai CY, Wen SY, Shibu MA, Yang YC, Peng H, Wang B, Wei YM, Chang HY, Lee CY, Huang CY, Kuo WW. Diallyl trisulfide protects against high glucose-induced cardiac apoptosis by stimulating the production of cystathionine gamma-lyase-derived hydrogen sulfide. Int J Cardiol. 2015;195:300–10.

46. Shibu MA, Kuo CH, Chen BC, Ju DT, Chen RJ, Lai CH, Huang PJ, Viswanadha VP, Kuo WW, Huang CY. Oolong tea prevents cardiomyocyte loss against hypoxia by attenuating p-JNK mediated hypertrophy and enhancing P-IGF1R, p-akt, and p-bad (ser136) activity and by fortifying NRF2 antioxidation system. Environ Toxicol. 2018;33:220–33.

47. Asokan Shibu M, Kuo W-W, Kuo C-H, Day C-H, Shen C-Y, Chung L-C, Lai C-H, Pan L-F, Vijaya Padma V, Huang C-Y. Potential phytoestrogen alternatives exert cardio-protective mechanisms via estrogen receptors. BioMedicine. 2017;7:11.

48. Shibu MA, Agrawal DC, Huang C-Y. Mushrooms: A Pandora Box of Cardioprotective Phytochemicals. In: Agrawal DC, Tsay H-S, Shyur L-F, Wu Y-C, Wang S-Y, editors. Medicinal Plants and Fungi: Recent Advances in Research and Development. Singapore: Springer Singapore; 2017. p. 337–62.

49. Chiang JT, Badrealam KF, Shibu MA, Kuo CH, Huang CY, Chen BC, Lin YM, Viswanadha VP, Kuo WW, Huang CY. Eriobotrya japonica ameliorates cardiac hypertrophy in H9c2 cardiomyoblast and in spontaneously hypertensive rats. Environ Toxicol. 2018;33:1113–22.

50. Lin K-H, Shibu MA, Peramaiyan R, Chen Y-F, Shen C-Y, Hsieh Y-L, Chen R-J, Viswanadha VP, Kuo W-W, Huang C-Y. Bioactive flavone fisetin attenuates hypertension associated cardiac hypertrophy in H9c2 cells and in spontaneously hypertension rats. J Funct Foods. 2019;52:212–8.

51. Pucciarelli DL. Cocoa and heart health: a historical review of the science. Nutrients. 2013;5:3854–70.

52. Magi E, Bono L, Di Carro M. Characterization of cocoa liquors by GC-MS and LC-MS/MS: focus on alkylpyrazines and flavanols. J Mass Spectrom. 2012;47:1191–7.

53. Chen J, Tian J, Ge H, Liu R, Xiao J. Effects of tetramethylpyrazine from Chinese black vinegar on antioxidant and hypolipidemia activities in HepG2 cells. Food Chem Toxicol. 2017;109:930–40.

54. Chen Z, Pan X, Georgakilas AG, Chen P, Hu H, Yang Y, Tian S, Xia L, Zhang J, Cai X, et al. Tetramethylpyrazine (TMP) protects cerebral neurocytes and inhibits glioma by down regulating chemokine receptor CXCR4 expression. Cancer Lett. 2013;336:281–9.

55. Chen HP, He M, Huang QR, Zeng GH, Liu D. Delayed protection of tetramethylpyrazine on neonatal rat cardiomyocytes subjected to anoxia-reoxygenation injury. Basic Clin Pharmacol Toxicol. 2007;100:366–71.

56. Gong X, Ivanov VN, Hei TK. 2,3,5,6-Tetramethylpyrazine (TMP) down-regulated arsenic-induced heme oxygenase-1 and ARS2 expression by inhibiting Nrf2, NF-kappaB, AP-1 and MAPK pathways in human proximal tubular cells. Arch Toxicol. 2016;90:2187–200.

57. Zhang X, Dong H, Liu Y, Han J, Tang S, Si J. Tetramethylpyrazine partially relieves hypoxia-caused damage of cardiomyocytes H9c2 by downregulation of miR-449a. J Cell Physiol. 2019;234:15098–107.

58. Li M, Zhao C, Wong RN, Goto S, Wang Z, Liao F. Inhibition of shear-induced platelet aggregation in rat by tetramethylpyrazine and salvianolic acid B. Clin Hemorheol Microcirc. 2004;31:97–103.

59. Li RW, Yang C, Shan L, Zhang Z, Wang Y, Kwan YW, Lee SM, Hoi MP, Chan SW, Cheung AC, et al. Relaxation effect of a novel Danshensu/tetramethylpyrazine derivative on rat mesenteric arteries. Eur J Pharmacol. 2015;761:153–60.

Publisher's Note