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ORIGINAL ARTICLE

Protective Effect of Ferulic Acid on Human Umbilical Vein Endothelial Cell Model of Cold Stress

Si Huang[1,2,†], Shengsuo Ma[1,†], Di Zhang[1], Bing Yang[1], Jianxin Sun[1], Meina Huang[1], Haoming Lin[1], Meijing Xie[1], Guoping Zhao[1]

[1]College of Traditional Chinese Medicine, Jinan University, Guangzhou, [2]Nanhai Hospital of Traditional Chinese Medicine, Foshan, Guangdong, China
[†]These authors contribute equally to this work

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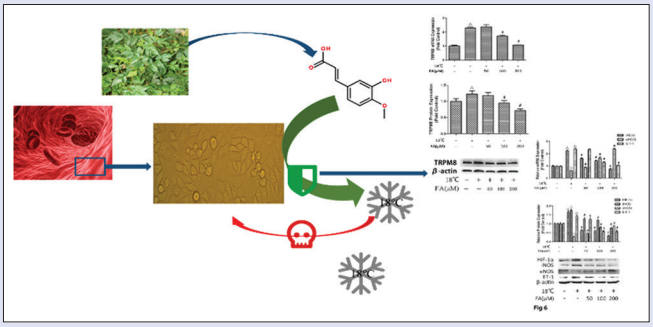
ABSTRACT

Context: Ferulic acid (FA) is an active principle derived from the traditional Chinese medicine *Angelica sinensis*, which has been used for the treatment of cardiovascular and cerebrovascular diseases in China for many years. However, a thorough understanding of effects on vascular function by FA has not been investigated. **Aims:** The aim of the present study was to investigate the potential mechanism of FA by suppressing Transient receptor potential cation channel subfamily M member 8 (TRPM8) channels and regulating endothelial nitric oxide (NO) pathway to ameliorate cold explore injury in human umbilical vascular endothelial cells (HUVECs). **Subjects and Methods:** The effects of cold exposure and FA on cell viability were detected by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay and lactate dehydrogenase (LDH) assay. Quantitative polymerase chain reaction and Western blot were utilized to detect TRPM8, hypoxia-inducible factor-alpha (HIF-1α), endothelin-1 (ET-1), inducible NO synthase (iNOS), endothelial NO synthase (eNOS) messenger RNA, and protein expression in HUVECs. Enzyme-linked immunosorbent assay method was used to detect the concentration of ET-1 in culture supernatants of HUVECs. **Results:** Cold exposure at 18°C had no significant effect on cell morphology but increased secretion of LDH and ET-1 and the expression of TRPM8, HIF-1α, iNOS, and ET-1. Treatment with FA decreased all of these changes. The levels of NO and eNOS decreased in cold stress model, while FA treatment attenuated the cold-induced decrease of NO and eNOS. **Conclusion:** Cold stress can cause an increase in vasoconstrictors such as TRPM8 and ET-1 and reducing cell viability, but FA can prevent cold stress-related cardiovascular disease by regulating the expression of these substances in cells.

Key words: Cold stress, endothelin-1, ferulic acid, transient receptor potential ion channel subfamily M member 8, vascular endothelial cell

SUMMARY

- Exposure of human umbilical vascular endothelial cells (HUVECs) to a temperature of 18°C increased transient receptor potential ion channel subfamily M member 8 (TRPM8) messenger RNA and disrupts the balance of the endothelin-nitric oxide (ET-NO) system
- FA treatment restores balance in the ET-NO system by increasing endothelial NO synthase expression and decreasing inducible NO synthase and ET-1 expression in VECs, resulting in vasodilation
- FA tends to restore TRPM8 expression to normal levels after low-temperature exposure
- FA might regulate the endothelium-mediated vasoconstriction and vasodilatation in part through the TRPM8/hypoxia-inducible factor-alpha/ET-1 signaling pathway.



Abbreviations used: FA: Ferulic acid; TRPM8: Transient receptor potential ion channel subfamily M member 8; HVECs: Human umbilical vein endothelial cells; ET-1: Endothelin-1; HIF-1α: Hypoxia-inducible factor-1α; eNOS: Endothelial nitric oxide synthase; iNOS: Inducible nitric oxide synthase; NO: Nitric oxide; CHD: Coronary heart disease; LDH: Lactate dehydrogenase.

Correspondence:

Prof. Guoping Zhao,
527 Room, College of Traditional Chinese Medicine,
Jinan University, Guangzhou,
Guangdong 510630, China.
E-mail: tguo428@jnu.edu.cn
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INTRODUCTION

Cold temperatures are closely linked to the morbidity and mortality of many types of diseases. Numerous studies have suggested that the mortality rate is much higher in winter than in other seasons and that seasonal mortality increases are greater among individuals with prevalent ischemic heart disease.[1-7] A worldwide population analysis shows that rates of coronary events increased during comparatively cold periods.[8] This is generally believed to be due to hypothermia and low-temperature activation of the body's, leading to severe vasoconstriction, resulting in tissue ischemia and metabolic disorders.[9,10] Endothelium injury or dysfunction may be a critical and initiating factor in this process by control of vasomotor tone by the release of vasoactive substances such as endothelin-1 (ET-1), hypoxia-inducible factor-alpha (HIF-1α), and

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NO. Recent studies show that transient receptor potential ion channel subfamily M member 8 is also widely expressed in the endothelium and vascular smooth muscles and on activation causes vasoconstriction or vasodilatation.[11,12] As the inner cellular lining of blood vessels, the vascular endothelium which regulates vascular tone, proliferation, and permeability of inflammatory inducers or infiltration of leukocytes is emphasized as the central spot of endothelium dysfunction.[13] Because of the similarity between human umbilical vein endothelial cells (HUVECs) and arterial endothelial cells, HUVEC is a common material to construct endothelial injury model.[14,15] Ferulic acid (FA) is a phenolic acid widely found in natural plants. It has many pharmacological effects such as inhibiting platelet aggregation,[16] releasing serotonin, scavenging free radicals,[17,18] fighting infection, and regulating immunity.[19] In China, FA is usually used in cold-related diseases such as coronary heart disease (CHD), Raynaud's syndrome, and vasculitis. Evidence suggests FA and its derivatives exert multiple effects on the formation of CHD, including the inhibition of coronary atherosclerosis, improvement vascular function, and protective effect of the myocardium.[20-23] In the past research, we have found that FA and other compositions in DangGui SiNi Decoction play a protective role in ischemia/reperfusion heart through regulating the endothelial nitric oxide synthase (eNOS)/NO signal pathway by increasing the PeNOS protein expression and decreasing the expression of inducible NO synthase (iNOS) protein, and FA also can regulate cold-induced vasospasm by inhibiting the expression of transient receptor potential ion channel subfamily M member 8 (TRPM8).[24,25] The goals of the study described here were as follows: (1) to explore the hypothesis that cold-mediated activation of the TRPM8 channel is responsible for an imbalance in the ET-NO system and (2) to determine the role of the TRPM8 channel in the molecular mechanisms of FA-mediated cardioprotection.

SUBJECTS AND METHODS

Cell viability assay

HUVECs were cultured in 96-well tissue culture plates (5×10^{3} cells/well) with 10% fetal bovine serum for 24 h and exposed to FA at different temperatures (37 or 18°C) for 5 h. Cell viability was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method according to the manufacturer's instructions (SIGMA Company, Continental, USA).

Determination of lactate dehydrogenase activity and NO and endothelin-1 expression

After exposure to FA at different temperatures (37°C or 18°C), the medium in individual wells was collected and centrifuged to remove dead cells. Lactate dehydrogenase (LDH) activity in the medium was measured using an LDH assay kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The NO and ET-1 levels in the supernatant were measured using an NO assay kit (Nanjing Jiancheng Bioengineering Institute, China) and an ET-1 enzyme-linked immunosorbent assay kit (Bioleaf Company, China), respectively, according to the manufacturer's instructions.

RNA isolation, reverse transcription, and real-time polymerase chain reaction analysis

Total RNA was extracted from the cells using the Trizol reagent (Takara, Japan) according to the manufacturer's instructions. Isolated RNA was reverse transcribed into complementary DNA (cDNA) using a high-capacity cDNA reverse transcription kit (Takara, Japan) according to the manufacturer's instructions and incubated in a gradient polymerase chain reaction (PCR) thermal cycler (Bio-Rad, USA). Gene expression of relative messenger RNA (mRNA) was monitored by quantitative real-time PCR using PrimeScript RT Master Mix (Takara, Japan) and a PCR instrument for fluorescence detection (Bio-Rad, USA). The amplification cycles consisted of denaturation at 95°C for 2 min, 40 cycles of denaturation at 95°C for 5 s, annealing at 58°C for 30 s (eNOS/ET-1 is annealing at 60°C for 30 s). The sequences of the specific primers were as follows: TRPM8 (sense: 5`-CAGCACTGGCACCTGAAAAC-3` and antisense: 5`-GGACTGCGCGATGTAGATGA-3`), iNOS (sense: 5`-GAGCATCACCCCCGTGTTTCA-3` and antisense: 5`-TCTTGGGTCTCCGCTTCTCGTC-3`), eNOS (sense: 5`-CGAGTGAAGGCGACAATCCT-3` and antisense: 5`-CGAGGGACACCACGTCATAC-3`), ET-1 (sense: 5`-GAAACCCACTCCCAGTCCAC-3` and antisense: 5`-TCTTGGACCTAGGGCTTCCA-3`), and β-actin (sense: 5`-GGGAAATCGTGCGTGACATTAAGG-3` and antisense: 5`-CAGGAAGGAAGGCTGGAAGAGTG-3`).

Western blot analysis

After exposure to different stimulation conditions for 5 h, the cells were laced with an equal volume of radioimmunoprecipitation assay buffer. The protein concentration was determined using the bicinchoninic acid assay. Total cellular protein (30 μg) was subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking with Tris-buffered saline with Tween-20 containing 5% nonfat milk for 1 h, the membrane was probed with primary antibodies against TRPM8 (1:1000; Abcam, #ab104569), HIF-1α (1:1000; Cell Signaling, #3716), iNOS (1:500; Novus Company, #NBP2-22119), eNOS (1:500; Cell Signaling, #5880S), ET-1 (1:1000; Novus Company, #NB300-526), and β-actin (1:200; Boster Company, #BM0627) overnight at 4°C. After incubation with the appropriate secondary antibody for 1 h, BeyoECL Plus (Beyotime Biotechnology) was used for detection.

Statistical analysis

All data are shown as the means ± standard deviations. Data analyses were finished with SPSS version 19.0 software (IBM, USA). Measurement data in multiple groups were compared with two-way ANOVA followed by the Student–Newman–Keuls or Tamhane's *post hoc* test to assess the significance of differences between groups. $P < 0.05$ was considered statistically significant.

RESULTS

The temperature-mediated effects on cellular morphology are presented in Figure 1. HUVECs in the blank control group maintained a paved stone-like structure with closely packed cells and serrated edges that fit next to each other. Similar cell morphology was observed in the model-alone group cultured at 18°C.

The effects of different temperatures and drug on LDH activity in the growth medium are presented in Figure 2. Compared to the blank control group, the model-alone groups exhibited significant increases in LDH activity ($P < 0.05$). Lower temperatures produced greater increases in LDH activity. Compared to the model-alone group at 18°C, the medium- and high-dose FA groups exhibited significant decreases in LDH activity ($P < 0.05$).

The effects of different temperatures and drug treatment on cell viability are presented in Figure 3. No differences in cell viability were observed in any experimental group cultured at 18°C.

The effects of different temperatures and drug treatments on ET-1 and NO secretion levels are presented in Figure 4. Compared to the

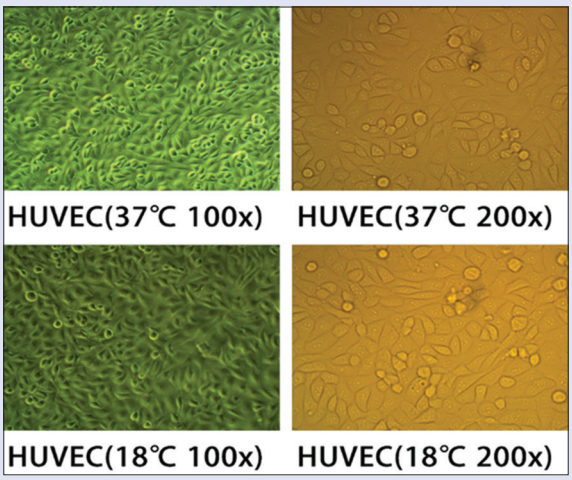

Figure 1: Cell morphology at different temperatures

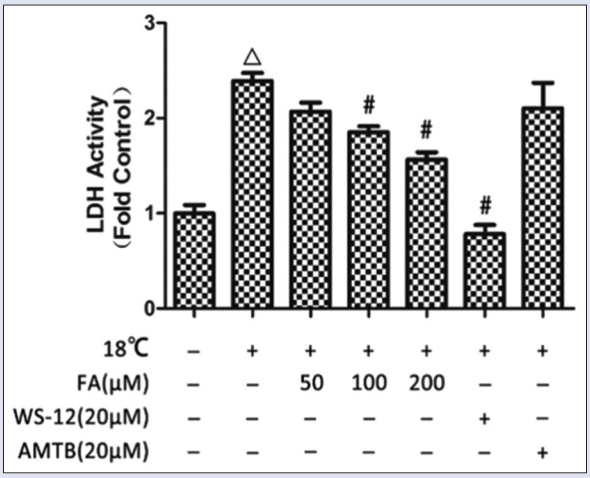

Figure 2: Influence of 18°C, ferulic acid, and transient receptor potential ion channel subfamily M member 8 to lactate dehydrogenase activity in the medium; data were shown as means ± standard deviations (n = 4). Δ: P < 0.05 versus untreated control, #: P < 0.05 versus 18°C group

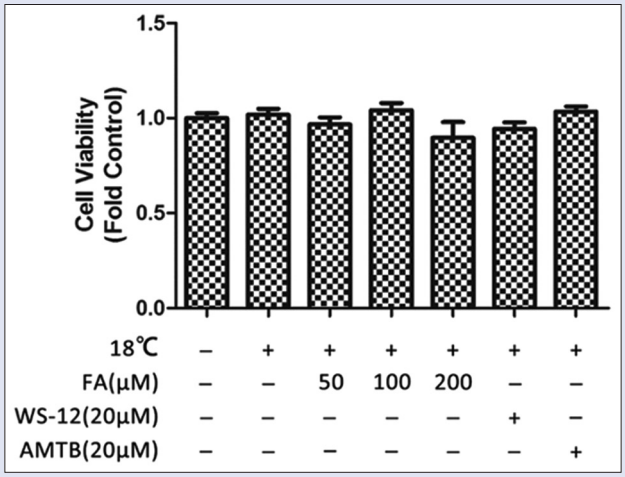

Figure 3: Influence of 18°C, ferulic acid, and transient receptor potential ion channel subfamily M member 8 to cell viability in human umbilical vascular endothelial cells; data were shown as means ± standard deviations (n = 5). Δ: P < 0.05 versus untreated control, #: P < 0.05 versus 18°C group

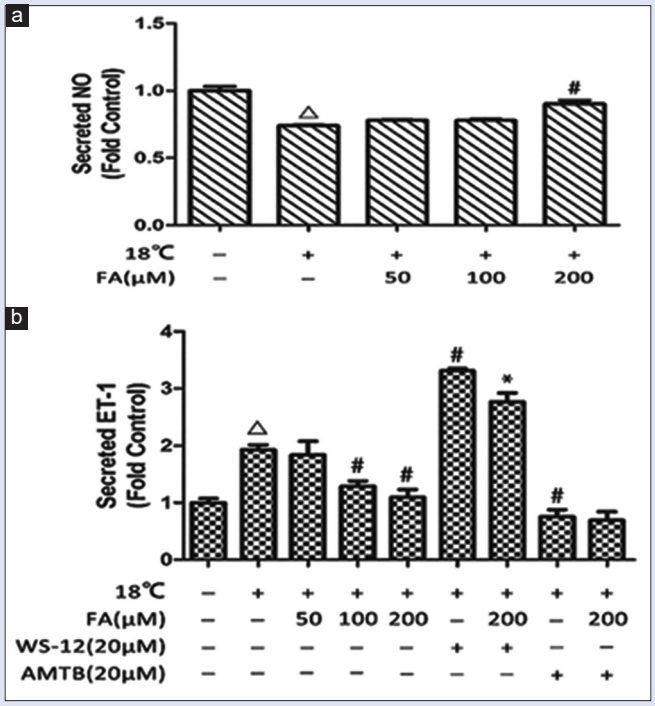

Figure 4: Influence of 18°C and ferulic acid and transient receptor potential ion channel subfamily M member 8 to endothelin-1 and nitric oxide secretions in the medium. Data were shown as means ± standard deviations. Δ: P < 0.05 versus untreated control, #: P < 0.05 versus 18°C group, *: P < 0.05 versus 18°C + WS-12 20-μM group. (a) Nitric oxide secretions of different groups (n = 3); (b) Endothelin-1 secretions of different groups (n = 4)

blank control group, the ET-1 secretion of the model-alone group at 18°C was significantly increased (P < 0.05). Compared to the model-alone group at 18°C, the ET-1 and NO secretion levels of the FA were significantly decreased (P < 0.05). Compared to the blank control group, the NO secretion of the model-alone group at 18°C was significantly decreased (P < 0.05). Compared to the model-alone group at 18°C, the high-dose FA groups exhibited significantly increased NO secretion (P < 0.05).

The effects of different temperatures and FA treatment on the gene and protein expression levels of TRPM8 are presented in Figure 5. Compared to the blank control group, the model-alone group at 18°C exhibited significantly increased TRPM8 expression (P < 0.05). Compared to the model-alone group at 18°C, TRPM8 expression in the medium- and high-dose FA groups was significantly decreased (P < 0.05).

The effects of different temperatures and drug treatments on the expression levels of genes and proteins related to endothelial function are illustrated in Figures 6 and 7. Compared to the blank control group, the expression of iNOS, ET-1, and HIF-1α in the model-alone group at 18°C was significantly increased (P < 0.05), whereas the expression of eNOS in the model-alone group at 18°C was significantly decreased (P < 0.05). Compared to the model-alone group at 18°C, the expression levels of HIF-1α and ET-1 in the FA groups were significantly decreased (P < 0.05).

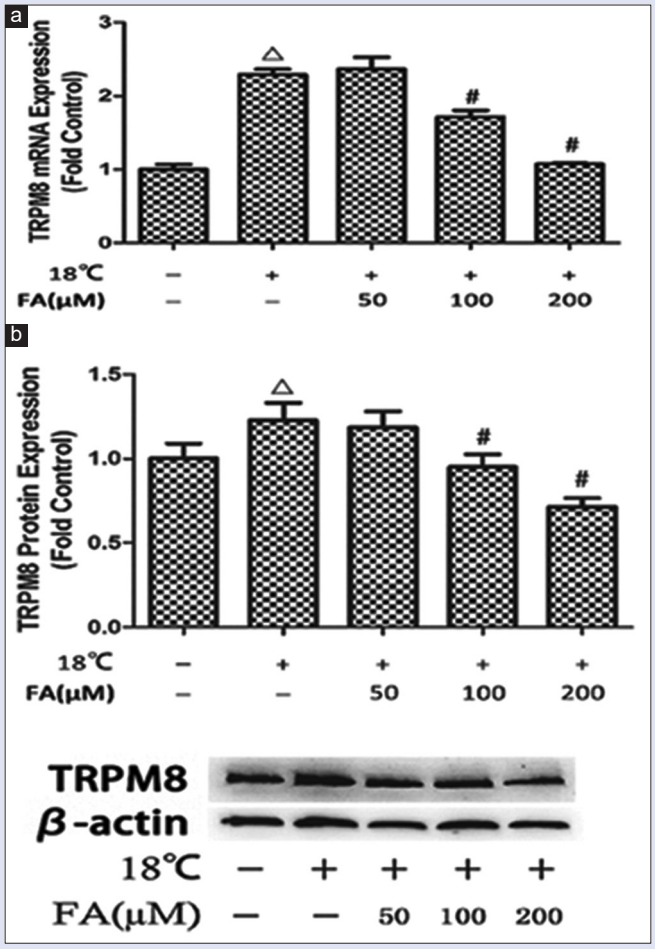

Figure 5: Influence of 18°C, ferulic acid to transient receptor potential ion channel subfamily M member 8 expressions. Data were shown as means ± standard deviations (n = 3). Δ: $P < 0.05$ versus untreated control. (a) Transient receptor potential ion channel subfamily M member 8 mRNA expressions were determined by quantitative polymerase chain reaction. (b) Transient receptor potential ion channel subfamily M member 8 protein expressions were determined by Western blot

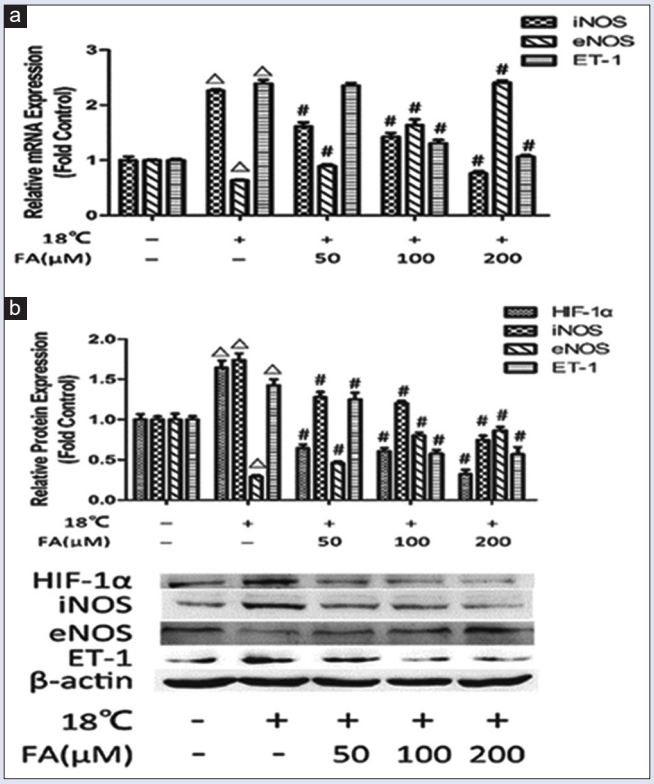

Figure 6: Influence of 18°C and ferulic acid to hypoxia-inducible factor-alpha, inducible nitric oxide synthase, endothelial nitric oxide synthase, and endothelin-1 expressions. Data were shown as means ± standard deviations (n = 3). Δ: $P < 0.05$ versus untreated control, #: $P < 0.05$ versus 18°C group. (a) Inducible nitric oxide synthase, endothelial nitric oxide synthase, and endothelin-1 messenger RNA expressions were determined by quantitative polymerase chain reaction. (b) Hypoxia-inducible factor-alpha, inducible nitric oxide synthase, endothelial nitric oxide synthase, and endothelin-1 proteins expressions were determined by Western blot

DISCUSSION

Considerable interest has been raised in the causes and mechanisms of exacerbation of CHD because such exacerbations are important causes of morbidity and mortality in CHD patients. Low ambient temperatures might contribute to CHD exacerbations through the induction of vascular contraction, leading to coronary spasm stenosis and myocardial ischemia. These conditions may cause angina pectoris or even acute coronary syndrome. Low ambient temperatures are combined with an increased frequency of exacerbations in CHD patients.

TRPM8 channels are present in mammal VECs[11] and play an important role in the pathogenesis of numerous cold-related diseases. Low temperatures and chemical cooling agents increase bronchial epithelial cell TRPM8 expression which induces airway responsiveness. TRPM8 channels expressed in VECs may act as sensory receptors and participate in endothelium-mediated vasoconstriction induced by low ambient temperatures. Here, we have found that exposure of HUVECs to a temperature of 18°C increased TRPM8 mRNA and protein expression levels, and this increase was inhibited in a dose-dependent manner by treatment with FA.

VECs regulate vasoconstriction mainly through the ET-NO system which is also associated with inflammation, thrombosis, and vascular elasticity. ET-1 concentrations in the blood of rats increase during the first few days of cold exposure.[26] Increased ET-1 expression plays an important role in cold-induced diseases. Excessive ET-1 secretion causes local disturbances of the microcirculation, ischemia, and hypoxia, leading to inflammation and cell necrosis. NO is generated by NOS in mammals. The major NOSs in VECs consist of iNOS and eNOS. NO, which is secreted by eNOS, can not only vasodilate vessels but also inhibit platelet aggregation, ET-1 synthesis, and thrombosis.[27] iNOS, which is mildly expressed under normal physiological conditions, increases in expression following cellular injury. Excessive iNOS production leads to cytotoxicity, which induces inflammation, oxidative stress, and cell necrosis. Our research has shown that low ambient temperatures upregulate iNOS and ET-1 expression, downregulate eNOS expression, decrease NO secretion, increase ET-1 secretion, and lead to vasoconstriction. FA treatment restores balance in the ET-NO system by increasing eNOS expression and decreasing iNOS and ET-1 expression in VECs, resulting in vasodilation.

Low ambient temperatures disrupt the balance of the ET-NO system. We determined whether the TRPM8 channel is responsible for imbalances in the ET-NO system. Calmodulin-dependent kinase (CaMK), which is regulated by intracellular Ca^{2+} concentrations,

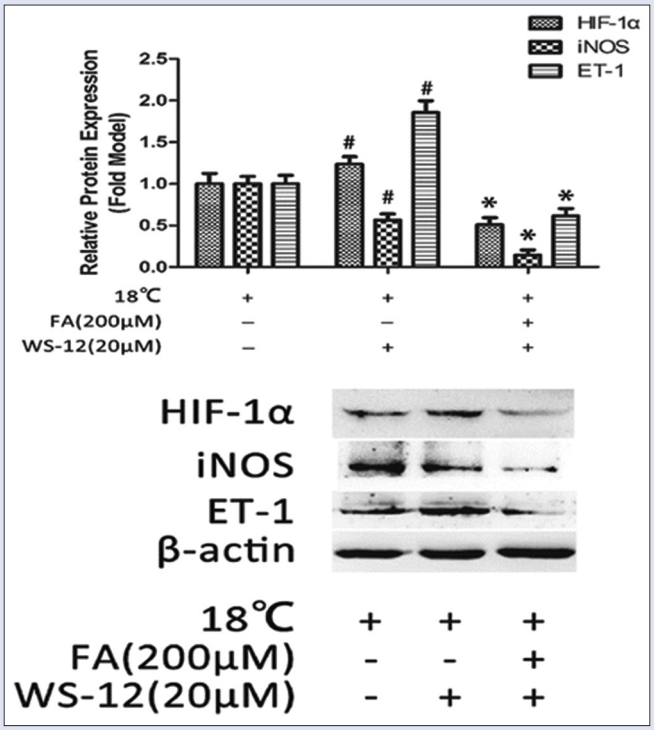

Figure 7: Influence of transient receptor potential ion channel subfamily M member 8 agonist WS-12 to hypoxia-inducible factor-alpha, endothelin-1, and inducible nitric oxide synthase, all proteins levels were determined by Western blot. Data were shown as means ± standard deviations (n = 3). Δ: $P < 0.05$ versus untreated control, #: $P < 0.05$ versus 18°C group

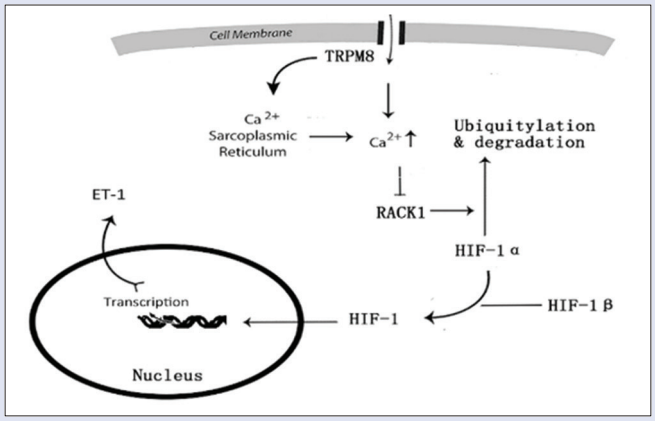

Figure 8: Sketch map of synthesis of endothelin-1 mediated by transient receptor potential ion channel subfamily M member 8. Under the low temperature situation, activated TRPM8 channels induce Ca^{2+} influx which suppresses the expression of RACK-1 and promote activation of HIF-1. Activated HIF-1 enters the nucleus to initiate mRNA transcription of ET-1

regulates the expression of many proteins, including iNOS. Reducing intracellular Ca^{2+} concentrations inhibits the activity of CaMK and downregulates iNOS expression.[28] WS-12, a derivative of menthol and a TRPM8-specific agonist, activated TRPM8 channels to induce Ca^{2+} influx, leading to dowregulation of iNOS expression and secretion of NO. The increased iNOS expression induced in culture at 18°C was not mediated by TRPM8.

HIF-1α is the active subunit of HIF-1, which is a heterodimer consisting of HIF-1α and HIF-1β. Normally, HIF-1α would be degraded by ubiquitination as soon as translation is initiated. HIF-1 activity could be regulated by an O_2-independent but Ca^{2+}-dependent mechanism involving HIF-1α interaction with a scaffolding protein, RACK1.[29-33] Activated HIF-1 enters the nucleus to initiate mRNA transcription of downstream genes such as eNOS, iNOS, and ET-1. It has been shown that overexpressed TRPM8 suppresses RACK-1 dimerization, promoting activation of HIF-1 in prostate cancer cells.[34] In sickle cell anemia model mice, cold allodynia was related to acute VASO occlusion and caused pain; besides, the transcription levels of pain genes such as TRPM8 and ET-1 were significantly increased.[35] The expressions of HIF-1α in mammals were correlated with temperature, and it was found that HIF-1α involved in regulation of bitter pain sensitivity and continuous in mice by gene knockout technique.[36] Our research showed simultaneous induction of TRPM8, HIF-1α, and ET-1 expression induced by culture at 18°C and FA treatment. We speculated that TRPM8 channels regulate cold-induced ET-1 expression through HIF-1α. FA might regulate the endothelium mediated vasoconstriction and vasodilatation in part through the TRPM8/HIF-1α/ET-1 signaling pathway, as shown in Figure 8.

Very low ambient temperatures disrupt cell membrane integrity and intracellular proteins, which significantly inhibit the normal cellular metabolism.[37] The cell membrane has a large surface area and is highly water permeable. Low ambient temperatures lead to severe cellular dehydration and cell shrinkage. The increased concentration of intracellular electrolytes induced by dehydration damages cell membranes and intracellular proteins. The cell membrane transforms from liquid crystals to gel molecules as dehydration progresses. Direct structural changes to the cell membrane lead to increasing cell membrane permeability, loss control over exchange of internal and external material, and leakage of soluble substances into the cell, which cause cell damage. However, electrolyte concentrations deactivate some intracellular damage-activated proteins, especially key enzymes. The LDH activity in the medium could be used to estimate the membrane permeability, and our results showed that culture at 18°C damaged cell membranes. Further study has responsibility for determining whether the increase in NO secretion and inhibition of TRPM8 expressions related to membrane damage.

CONCLUSION

In summary, our study indicated that FA exerted protective effects on HUVECs model of cold stress through the TRPM8/HIF-1α/ET-1 signaling pathway. It raises the possibility that FA is a promising candidate for the development of anticardiovascular disease agents.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Mackenbach JP, Kunst AE, Looman CW. Seasonal variation in mortality in the Netherlands. J Epidemiol Community Health 1992;46:261-5.

2. Crombie DL, Fleming DM, Cross KW, Lancashire RJ. Concurrence of monthly variations of mortality related to underlying cause in Europe. J Epidemiol Community Health 1995;49:373-8.
3. Gorjanc ML, Flanders WD, VanDerslice J, Hersh J, Malilay J. Effects of temperature and snowfall on mortality in Pennsylvania. Am J Epidemiol 1999;149:1152-60.
4. van Rossum CT, Shipley MJ, Hemingway H, Grobbee DE, Mackenbach JP, Marmot MG. Seasonal variation in cause-specific mortality: Are there high-risk groups? 25-year follow-up of civil servants from the first whitehall study. Int J Epidemiol 2001;30:1109-16.
5. Bruno RM, Taddei S. ' 'tis bitter cold and I am sick at heart': Establishing the relationship between outdoor temperature, blood pressure, and cardiovascular mortality. Eur Heart J 2015;36:1152-4.
6. Ryti NR, Guo Y, Jaakkola JJ. Global association of cold spells and adverse health effects: A systematic review and meta-analysis. Environ Health Perspect 2016;124:12-22.
7. Ikäheimo TM. Cardiovascular diseases, cold exposure and exercise. Temperature (Austin) 2018;5:123-46.
8. Barnett AG, Dobson AJ, McElduff P, Salomaa V, Kuulasmaa K, Sans S, *et al.* Cold periods and coronary events: An analysis of populations worldwide. J Epidemiol Community Health 2005;59:551-7.
9. Heindl S, Struck J, Wellhöner P, Sayk F, Dodt C. Effect of facial cooling and cold air inhalation on sympathetic nerve activity in men. Respir Physiol Neurobiol 2004;142:69-80.
10. Muller MD, Gao Z, Drew RC, Herr MD, Leuenberger UA, Sinoway LI. Effect of cold air inhalation and isometric exercise on coronary blood flow and myocardial function in humans. J Appl Physiol (1985) 2011;111:1694-702.
11. Johnson CD, Melanaphy D, Purse A, Stokesberry SA, Dickson P, Zholos AV. Transient receptor potential melastatin 8 channel involvement in the regulation of vascular tone. Am J Physiol Heart Circ Physiol 2009;296:H1868-77.
12. Zholos A, Johnson C, Burdyga T, Melanaphy D. TRPM channels in the vasculature. In: Islam MS, editor. Transient Receptor Potential Channels. Advances in Experimental Medicine and Biology. Vol. 704. Berlin: Springer-Verlag Berlin; 2011. p. 707-29.
13. Aird WC. Phenotypic heterogeneity of the endothelium: II. Representative vascular beds. Circ Res 2007;100:174-90.
14. Chen WL, Huang XQ, Zhao LY, Li J, Chen JW, Xiao Y, *et al.* Involvement of kv1.5 protein in oxidative vascular endothelial cell injury. PLoS One 2012;7:e49758.
15. Gao P, Zhao Z, Zhang C, Wang C, Long K, Guo L, *et al.* The therapeutic effects of traditional Chinese medicine Fusu agent in LPS-induced acute lung injury model rats. Drug Des Devel Ther 2018;12:3867-78.
16. Hou YZ, Yang J, Zhao GR, Yuan YJ. Ferulic acid inhibits vascular smooth muscle cell proliferation induced by angiotensin II. Eur J Pharmacol 2004;499:85-90.
17. Mathew S, Abraham TE. Ferulic acid: An antioxidant found naturally in plant cell walls and feruloyl esterases involved in its release and their applications. Crit Rev Biotechnol 2004;24:59-83.
18. Srinivasan M, Sudheer AR, Menon VP. Ferulic acid: Therapeutic potential through its antioxidant property. J Clin Biochem Nutr 2007;40:92-100.
19. Salazar-López NJ, Astiazarán-García H, González-Aguilar GA, Loarca-Piña G, Ezquerra-Brauer JM, Domínguez Avila JA, *et al.* Ferulic acid on glucose dysregulation, dyslipidemia, and inflammation in diet-induced obese rats: An integrated study. Nutrients 2017;9. pii: E675.
20. Jin Son M, Rico CW, Hyun Nam S, Young Kang M. Influence of oryzanol and ferulic acid on the lipid metabolism and antioxidative status in high fat-fed mice. J Clin Biochem Nutr 2010;46:150-6.
21. Tsai YC, Lee YM, Hsu CH, Leu SY, Chiang HY, Yen MH, *et al.* The effect of ferulic acid ethyl ester on leptin-induced proliferation and migration of aortic smooth muscle cells. Exp Mol Med 2015;47:e180.
22. Senaphan K, Kukongviriyapan U, Sangartit W, Pakdeechote P, Pannangpetch P, Prachaney P, *et al.* Ferulic acid alleviates changes in a rat model of metabolic syndrome induced by high-carbohydrate, high-fat diet. Nutrients 2015;7:6446-64.
23. Gao Y, Hao J, Zhang H, Qian G, Jiang R, Hu J, *et al.* Protective effect of the combinations of glycyrrhizic, ferulic and cinnamic acid pretreatment on myocardial ischemia-reperfusion injury in rats. Exp Ther Med 2015;9:435-45.
24. Qian GQ, Ding J, Zhang X, Yin X, Gao Y, Zhao GP. Preconditioning with glycyrrhizic, ferulic, paeoniflorin, cinnamic prevents rat hearts from ischemia/reperfusion injury via endothelial nitric oxide pathway. Pharmacogn Mag 2015;11:292-6.
25. Pan Y, Zhao G, Cai Z, Chen F, Xu D, Huang S, *et al.* Synergistic effect of ferulic acid and Z-ligustilide, major components of *A. Sinensis*, on regulating cold-sensing protein TRPM8 and TPRA1 *in vitro*. Evid Based Complement Alternat Med 2016:3160247.
26. Sun Z. Cardiovascular responses to cold exposure. Front Biosci (Elite Ed) 2010;2:495-503.
27. Gkaliagkousi E, Ferro A. Nitric oxide signalling in the regulation of cardiovascular and platelet function. Front Biosci (Landmark Ed) 2011;16:1873-97.
28. Zhang B, Crankshaw W, Nesemeier R, Patel J, Nweze I, Lakshmanan J, *et al.* Calcium-mediated signaling and calmodulin-dependent kinase regulate hepatocyte-inducible nitric oxide synthase expression. J Surg Res 2015;193:795-801.
29. Mottet D, Michel G, Renard P, Ninane N, Raes M, Michiels C. Role of ERK and calcium in the hypoxia-induced activation of HIF-1. J Cell Physiol 2003;194:30-44.
30. Salnikow K, Kluz T, Costa M, Piquemal D, Demidenko ZN, Xie K, *et al.* The regulation of hypoxic genes by calcium involves c-jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Mol Cell Biol 2002;22:1734-41.
31. Hui AS, Bauer AL, Striet JB, Schnell PO, Czyzyk-Krzeska MF. Calcium signaling stimulates translation of HIF-alpha during hypoxia. FASEB J 2006;20:466-75.
32. Tajima N, Schönherr K, Niedling S, Kaatz M, Kanno H, Schönherr R, *et al.* Ca2+-activated K+channels in human melanoma cells are up-regulated by hypoxia involving hypoxia-inducible factor-1alpha and the von hippel-lindau protein. J Physiol 2006;571:349-59.
33. Wang J, Weigand L, Lu W, Sylvester JT, Semenza GL, Shimoda LA. Hypoxia inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular ca2+in pulmonary arterial smooth muscle cells. Circ Res 2006;98:1528-37.
34. Yu S, Xu Z, Zou C, Wu D, Wang Y, Yao X, *et al.* Ion channel TRPM8 promotes hypoxic growth of prostate cancer cells via an O2 -independent and RACK1-mediated mechanism of HIF-1α stabilization. J Pathol 2014;234:514-25.
35. Zappia KJ, Garrison SR, Hillery CA, Stucky CL. Cold hypersensitivity increases with age in mice with sickle cell disease. Pain 2014;155:2476-85.
36. Kanngiesser M, Mair N, Lim HY, Zschiebsch K, Blees J, Häussler A, *et al.* Hypoxia-inducible factor 1 regulates heat and cold pain sensitivity and persistence. Antioxid Redox Signal 2014;20:2555-71.
37. Han B, Bischof JC. Direct cell injury associated with eutectic crystallization during freezing. Cryobiology 2004;48:8-21.