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Protection of dilator function of coronary arteries from homocysteine by tetramethylpyrazine: Role of ER stress in modulation of BK_{Ca} channels

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ABSTRACT

Objectives: We recently reported the involvement of ER stress-mediated BK_{Ca} channel inhibition in homocysteine-induced coronary dilator dysfunction. In another study, we demonstrated that tetramethylpyrazine (TMP), an active ingredient of the Chinese herb Chuanxiong, possesses potent anti-ER stress capacity. The present study investigated whether TMP protects BK_{Ca} channels from homocysteine-induced inhibition and whether suppression of ER stress is a mechanism contributing to the protection. Furthermore, we explored the signaling transduction involved in TMP-conferred protection on BK_{Ca} channels.

Methods: BK_{Ca} channel-mediated relaxation was studied in porcine small coronary arteries. Expressions of BK_{Ca} channel subunits, ER stress molecules, and E3 ubiquitin ligases, as well as BK_{Ca} ubiquitination were determined in porcine coronary arterial smooth muscle cells (PCASMCs). Whole-cell BK_{Ca} currents were recorded.

Results: Exposure of PCASMCs to homocysteine or the chemical ER stressor tunicamycin increased the expression of ER stress molecules, which was significantly inhibited by TMP. Suppression of ER stress by TMP preserved the BK_{Ca} β1 protein level and restored the BK_{Ca} current in PCASMCs, concomitant with an improved BK_{Ca}-mediated dilatation in coronary arteries. TMP attenuated homocysteine-induced BK_{Ca} β1 protein ubiquitination, in which inhibition of ER stress-mediated FoxO3a activation and FoxO3a-dependent atrogin-1 and Murf-1 was involved.

Conclusions: Reversal of BK_{Ca} channel inhibition via suppressing ER stress-mediated loss of β1 subunits contributes to the protective effect of TMP against homocysteine on coronary dilator function. Inhibition of FoxO3a-dependent ubiquitin ligases is involved in TMP-conferred normalization of BK_{Ca} β1 protein level. These results provide new mechanistic insights into the cardiovascular benefits of TMP.

1. Introduction

Large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel or Maxi K expressed in smooth muscle cells is an important regulator of vascular tone. Opening of BK_{Ca} channels inhibits voltage-dependent Ca^{2+} channels, limiting extracellular Ca^{2+} entry in smooth muscle cells thereby suppressing constriction of the vessel [16]. BK_{Ca} channels are known as a target site of relaxing factors released from endothelium including nitric oxide (NO) [6,20] and endothelium-derived hyperpolarizing factor (EDHF) [2,33]. Structurally, vascular BK_{Ca} channels consist of four pore-forming α subunits encoded by KCNMA1 and four modulatory β subunits, in particular KCNMB1-encoded β1 [11].

Alterations in the expression or the activity of BK_{Ca} channels have been found to be associated with vascular dysfunction in various diseases, such as atherosclerosis [28], hypertension, diabetes, and metabolic syndrome [1,7,8,18,22]. In a recent in vitro study, we demonstrated that elevated homocysteine level, which is an independent risk factor for atherosclerotic disease [26], impairs the function of BK_{Ca} channels in coronary arteries [29]. The finding of homocysteine-induced inhibition of BK_{Ca} currents was consistent with previous reports

Abbreviations: 4-PBA, 4-phenylbutyric acid; GRP78, 78-kDa glucose-regulated protein; ATF6, Activating transcription factor 6; ER stress, Endoplasmic reticulum stress; eIF2α, Eukaryotic translation initiation factor 2α; IRE1, Inositol-requiring enzyme 1; BK_{Ca} channel, Large-conductance Ca^{2+}-activated K^+ channel; LAD, Left anterior descending artery; PCASMCs, Porcine coronary artery smooth muscle cells; PERK, Protein kinase RNA-like endoplasmic reticulum kinase; TMP, Tetramethylpyrazine

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[3,9]. Importantly, in this study, we for the first time demonstrated that endoplasmic reticulum (ER) stress mediates homocysteine-induced BK_{Ca} channel inhibition via decreasing β1 subunits of the channel, in which ubiquitin ligase-promoted BK_{Ca} β1 degradation plays a significant role [29].

Tetramethylpyrazine (TMP, molecular formula C8H12N2), a major alkaloid-type compound isolated from *Ligusticum chuanxiong* (Chuan Xiong), is known for its cardiovascular benefits. The cardiovascular protective effects of TMP have been attributed to its vasodilatory, anti-inflammatory, antioxidant, and Ca^{2+} homeostatic activities [13,17,23,36]. We recently demonstrated that TMP has potent anti-ER stress activity. In ER-stressed coronary artery endothelial cells, TMP exhibited comparable inhibitory effect as ER stress inhibitors on the activation of the unfolded protein response (UPR) sensors, and such inhibition significantly contributes to its protective effect on endothelial function in conditions related to hypertension [19]. In vasculature, compared to a considerable amount of data showing the benefit of TMP on endothelial cells, studies concerning the effect of TMP on smooth muscle are far from adequate, for example, whether and how TMP regulates smooth muscle BK_{Ca} channels in diseased conditions remains barely studied.

In this study, we therefore aimed to investigate the effect of TMP on homocysteine-induced BK_{Ca} channel dysfunction, with further attempt to understand the role of the anti-ER stress activity of this compound in the regulation of BK_{Ca} channels.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals and reagents used in this study were: U46619 (Cayman Chemical, USA); NS1619 (Tocris Biosciences, UK); Type II collagenase and papain (Worthington Biochemical Corp, USA); bovine serum albumin (BSA) (Amresco, USA); bradykinin, iberiotoxin, DL-Dithiothreitol (DTT), homocysteine, tunicamycin, tetramethylpyrazine (TMP), and 4-phenylbutyric acid (4-PBA) obtained from Sigma-Aldrich Inc., Germany. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin-streptomycin (P/S) purchased from Thermal Fisher Scientific Inc., USA.

2.2. Preparation of porcine coronary arteries

Fresh hearts of young adult pigs (~35 kg) were collected from a local slaughterhouse. Once excised, the hearts were placed in cold (4 °C), pre-oxygenated (95%O_2/5%CO_2) Krebs solution and immediately transferred to the laboratory. All experiments were in accordance with institutional guidelines. Small coronary arteries (300–500 μm in diameter) were dissected from the branches of the left anterior descending artery (LAD) and the surrounding connective tissues were carefully removed.

2.3. Primary culture of porcine coronary artery smooth muscle cells (PCASMCs)

Smooth muscle cells were enzymatically disassociated from porcine small coronary arteries and cultured as in our previous studies [14,29]. In brief, arteries were cut open longitudinally, scraped off endothelium, and dissected into 1 × 1 mm^2 strips. The endothelium-denuded strips were digested at 37 °C for 30 min in 2 ml of phosphate-buffered saline solution (PBS) that contains 2 mg/ml of collagenase type II, 0.5 mg/ml of papain, 1.75 mg/ml of DTT, and 5 mg/ml BSA. After 5-min centrifugation at 1600 rpm, cells were re-suspended in 12 ml DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were then seeded and allowed to grow in a culture plate for 1 h at 37 °C before the culture medium was replaced to remove unattached cells. Attached PCASMCs were further cultured at 37 °C for approximately 1 week. For maintaining electrophysiological properties of isolated PCASMCs, only primary cultured cells were used for experiments.

2.4. Patch-clamp recording of BK_{Ca} channel currents in PCASMCs

K^+ currents of PCASMCs were recorded in a whole-cell mode by patch-clamp (EPC10, HEKA, Lambrecht, Germany) with further differentiation of the BK_{Ca} component, as we have reported elsewhere [14,29]. Solution used to fill the patch pipettes (3–5 MΩ resistance) was composed of (mmol/l): NaCl 10, KCl 110, $MgCl_2$ 4, $CaCl_2$ 7, ethyleneglycol tetraacetic acid (EGTA) 10, Mg-ATP 5, and HEPES 10. The bath solution contained (mmol/l): NaCl 130, KCl 5, $MgCl_2$ 1.2, $CaCl_2$ 1.5, Glucose 10, and HEPES 10. PCASMCs were held at −60 mV and voltage steps ranging from −100 to +100 mV were applied in 20-mV step increments for 500 milliseconds. Basal K^+ currents and the current in response to the BK_{Ca} channel activator NS1619 (3 μmol/l) were recorded. Iberiotoxin (100 nmol/l) was further applied to identify the BK_{Ca} current. Data were analyzed with PulseFit software (HEKA). The current was normalized by cell capacitance into current densities (pA/pF). All experiments were performed at room temperature (20–24 °C).

2.5. Isometric force study

Small coronary arteries were cut into 2-mm segments and denuded of endothelium before being mounted in a four-channel Mulvany myograph (Model 610 M, J.P.Trading, Aarhus, Denmark). The details of myograph study have been extensively published by our group [29,38]. Successful removal of endothelium was confirmed by the lack of relaxant response to endothelium-dependent vasodilators, as we previously reported [4,10]. Cumulative dose-response curve to the BK_{Ca} activator NS1619 (−8~−5 Log M) were then established following U46619 precontraction in the endothelium-denuded rings.

2.6. Reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Extraction of total RNA from PCASMCs using total RNA reagent (RNAiso Plus, TaKaRa Biotechnology, China) and conversion of mRNA to cDNA using reverse transcriptase kit (PrimeScript™ RT Master Mix, TaKaRa Biotechnology, China) were performed according to manufacturer's instructions. Amplification was performed using Taq DNA polymerase (GoTaq® G2 Flexi DNA Polymerase, Promega, USA) in the optimal condition for a PCR: 95 °C for 30s, 30 cycles at 95 °C for 30s, 50 °C for 1 min, 72 °C for 1 min, and 72 °C for 5 min. PCR products were separated by 1.5% agarose gel electrophoresis, and the density of the signals was analyzed by the Quantity One software (Bio-Rad Laboratories, Inc., USA). The following primers were used for PCR amplification: BK_{Ca} α, 5′-GCCAGCAACTTTCACTAC-3′ (forward) and 5′-CTGACAGGATAACGCACA-3′ (reverse); BK_{Ca} β1, 5′-TGTGCTGTCATCGCCTACT-3′ (forward) and 5′-ACCTGGTGCTCGTGGAAC-3′ (reverse); atrogin-1, 5′-TGGATGGCTGGGGATACAGA-3′ (forward) and 5′-TAAATTCCCCGCCAGTGTCC-3′ (reverse); Murf-1, 5′-GTGGTCATCTTGCCGTGTC-3′ (forward) and 5′-TGGTCCAGTAGGGATTTGC-3′ (reverse). GAPDH was amplified in parallel as an internal loading control with the primers 5′-GGTCGGAGTGAACGGATTT-3′ (forward) and 5′-ATTTGATGTTGGCGGGAT-3′ (reverse).

2.7. Western blot analysis

2.7.1. Protein expression at whole cell level

Whole cell protein of PCASMCs was extracted, fractionated, and electro-transferred to polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, USA) for detection of the expression of target proteins. In brief, PCASMCs were lysed in lysis buffer containing protease and phosphatase inhibitor cocktail (Roche Diagnostic, Switzerland) and centrifuged to collect protein. The protein extract was

then fractionated by a denaturing 8% sodium-dodecyl-sulfate polyacrylamidegel electrophoresis (30 μg per lane) for 90 min at 120 V and electro-transferred to PVDF membrane for 90 min at 100 V. The membrane was blocked with 5% non-fat milk/TBST for 1 h at room temperature and incubated in 5% non-fat milk/TBST overnight at 4 °C with specific primary antibody targeting 1) BK_{Ca} α (1:1000 dilution, Abcam) and BK_{Ca} β1 (1:500, Abcam); 2) ER stress molecules including 78-kDa glucose regulated protein (GRP78, 1:1000, Abcam), protein kinase RNA-like ER kinase (PERK, 1:1000, Cell Signaling), phosphorylated (Thr980) PERK (1:500, Bioss), inositol-requiring enzyme 1 (IRE1, 1:1000, Abcam), phosphorylated (Ser724) IRE1 (1:1000, Abcam), activating transcription factor 6 (ATF6, 1:1000, Abnova), eukaryotic translation initiation factor 2α ($eIF_{2\alpha}$, 1:1000, Abcam) and phosphorylated (Ser51) $eIF_{2\alpha}$ (1:500, Abcam); 3) transcription factor FoxO3a (1:500, Abcam); and 4) ubiquitin ligases including atrogin-1 (1:500, Abcam) and Murf-1 (1:500, Abnova). The membrane was then washed in TBST followed by incubation with secondary IRDye800®-infrared fluorescent dye-conjugated goat anti-rabbit or rabbit anti-mouse antibody (1:10000, Rockland) in TBST for 1 h at room temperature. Imaging was performed at a wavelength of 800 nm by using Odyssey gel imaging scanner (Li-Cor Biosciences). The intensity of the bands was analyzed by Quantity One imaging system (Version 4.6.6, Bio-Rad) and β-tubulin (1:2500, Abcam) was used as internal loading control.

2.7.2. Protein expression at subcellular level

Nuclear and cytoplasmic fractions of PCASMCs were separated by using NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, USA). The details of the method was described in our previous study [29]. PCASMCs were harvested with trypsin-EDTA (Thermo Fisher Scientific, USA) and centrifuged at 500 ×*g* for 5 min to collect the cell pellet. After wash, ice-cold cytoplasmic extraction reagents I and II were added to the cell pellet in proportion of 200:11 (μl). The supernatant was collected as cytoplasmic lysate after 5-min centrifugation at 16,000 ×*g*. Nuclear extraction reagent (100 μl) was then added to the precipitate and centrifuged at 16,000 ×*g* for another 10 min to collect the supernatant, the nuclear lysate. Proteins from nuclear and cytoplasmic preparations were examined for FoxO3a expression by Western blotting, with Lamin B1 (1:2000, Abcam) and β-tubulin (1:2500, Abcam) used as nuclear and cytoplasmic loading control respectively.

2.8. Protein knockdown by gene silencing

Three pairs of Murf-1 siRNAs were designed and synthesized for transfecting into PCASMCs: siMurf-1-1: 5′-GCCUGGAGAUGUUUACCA ATT-3′ (sense), 5′-UUGGUAAACAUCUCCAGGCTT-3′(antisense); siMurf-1-2: 5′-GCCAGAAAUUUGAUGUGCUTT-3′ (sense), 5′-AGCACA UCAAAUUUCUGGCTT-3′ (antisense); siMurf-1-3: 5′-GCAGCUCAUCAG AAGUAUUTT-3′ (sense), 5′-AAUACUUCUGAUGAGCUGCTT-3′ (antisense). PCASMCs were seeded in 6-well plate and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin until 70–80% confluence was reached. Cells were then incubated in Opti-MEM® I Reduced Serum Media (Thermo Fisher Scientific, USA) for 24 h. The mixture of siRNA (gene-specific or corresponding scrambled control) (20 μmol/L, Shanghai GenePharma, China) and Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific, USA) in proportion of 5:2 was added. After 6 h incubation, culture medium was replaced with normal DMEM and siRNA knockdown efficiency was evaluated by the mRNA and the protein level of Murf-1. The siRNA pair displaying the highest knockdown efficiency was chosen for subsequent experiments.

2.9. Ubiquitination assay

Total proteins extracted from PCASMCs underwent different treatments were immunoprecipitated by using Pierce Classic IP Kit (Thermo Fisher Scientific, USA). In brief, total proteins were harvested by IP lysis buffer with a BCA test to guarantee the final amount of total proteins no lower than 1000 μg. 10 μg of anti-BK_{Ca} β1 (1:50, Abcam) or rabbit IgG (1:50, Thermo Fisher Scientific, as negative control) was added into the cell lysates and incubated overnight at 4 °C. The immune complex was then pulled down by adding 10 μl of the Protein A/G agarose resin with gentle shaking for 1 h followed by 1000 ×*g* centrifugation. After washing, the precipitate was boiled for 5–10 min in 50 μl 2 × SDS PAGE sample buffer to release the IP products. The immunoprecipitated proteins were resolved on SDS-PAGE, followed by Western blot using anti-ubiquitin (1:1000, Cell Signaling) to determine the ubiquitination level of BK_{Ca} β1. Total cell lysate was used as the input control and rabbit IgG was used as the negative control.

2.10. Statistical analysis

Relaxation was expressed as the percentage decrease in isometric force induced by U46619. Expression of targets of interest was normalized to the expression of β-tubulin or Lamin B1 (for nuclear protein) for protein analysis, and to the level of GAPDH for mRNA analysis. Data were expressed as mean ± SEM. Statistical analyses were performed by one-way ANOVA followed by Scheffe tests (SPSS, version 20). $p < .05$ was considered statistically significant.

3. Results

3.1. TMP inhibits ER stress in PCASMCs subjected to homocysteine exposure

Homocysteine increased the expression of GRP78 and ATF6 and enhanced the phosphorylation of PERK, $eIF_{2\alpha}$, and IRE1 in PCASMCs, which is consistent with our previous data [29]. Compared to homocysteine-exposure, PCASMCs exposed to homocysteine while treated with TMP showed decreased expression and phosphorylation of these UPR sensor molecules, suggesting an inhibitory effect of TMP on homocysteine-induced ER stress in smooth muscle of coronary arteries (Fig. 1A & B).

The anti-ER stress activity of TMP was further demonstrated in PCASMCs exposed to tunicamycin, a chemical ER stress inducer. TMP significantly inhibited tunicamycin-induced upregulation of ATF6 and the phosphorylation of PERK and its downstream molecule $eIF_{2\alpha}$, and the inhibitory effect was comparable to that of the ER stress inhibitor 4-PBA (Fig. 1C & D). The concentrations of homocysteine (100 μmol/l), tunicamycin (5 μg/ml), and 4-PBA (2 mmol/l) used in this study remained the same as in our previous reports [19,29,32].

In normal PCASMCs, TMP itself showed no influence on the expression of ER stress-related molecules. The dosage of TMP (100 μmol/l) was chosen based on our previous study, in which we demonstrated that 100 μmol/l of TMP effectively protected endothelial cells from ER stress-induced injury in the same vasculature, and this concentration showed no cell toxicity through the 24 h culture period [19].

3.2. TMP protects BK_{Ca} channel function from impairment caused by homocysteine and chemical ER stress inducer in coronary arteries

The BK_{Ca} channel activator NS1619 induced dose-dependent relaxation in endothelium-denuded porcine coronary arteries. Exposure to homocysteine for 24 h attenuated the NS1619-induced vasorelaxation (Rmax: 57.7 ± 4.8% vs. 82.5 ± 2.8% in control, $p < .01$; EC50: -5.69 ± 0.10 vs. -6.20 ± 0.10 Log M, $p < .01$). Treatment with TMP restored the maximal relaxant response to 86.7 ± 3.7% ($p < .01$, vs. homocysteine) and left-shifted the EC50 to −5.98 ± 0.09 Log M ($p = .055$ vs. homocysteine) (Fig. 2A).

The inhibition of the relaxant response to NS1619 was also observed in coronary arteries subjected to tunicamycin exposure. As shown in

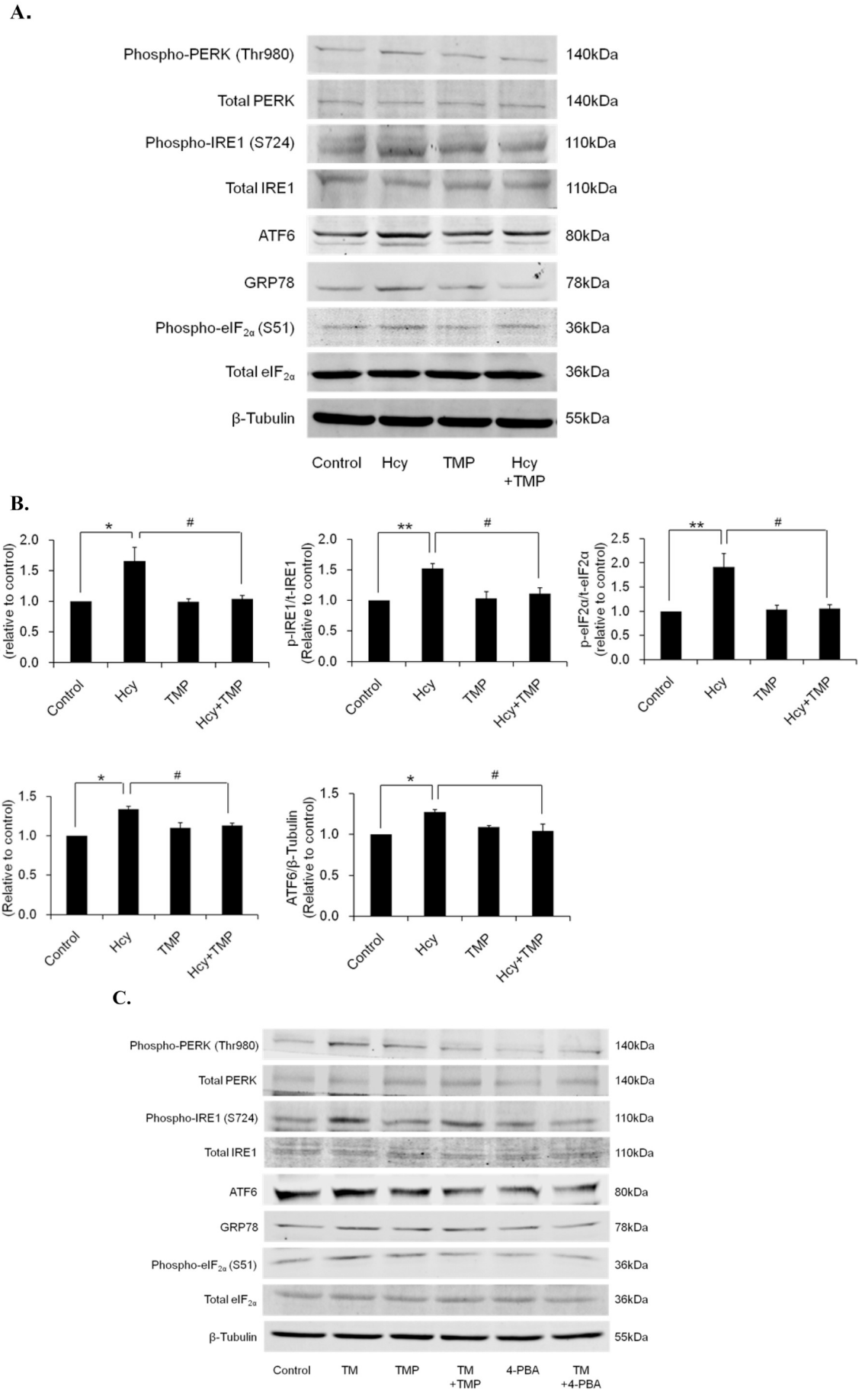

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Fig. 1. TMP inhibits homocysteine- (A & B) and tunicamycin- (C & D) induced ER stress in PCASMCs. A & C: Representative blots of ER stress molecules; B & D: Expression levels of ER stress molecules from 5 (B) and 7 (D) independent experiments.*$p < .05$, **$p < .01$ vs. control; #$p < .05$, ##$p < .01$ vs. Hcy or TM. TMP: tetramethylpyrazine, Hcy: homocysteine, TM: tunicamycin, 4-PBA: ER stress inhibitor.

Fig. 2B, relaxation induced by NS1619 was significantly attenuated at the concentration ranging from −6.5 to −5.5 logM, and such inhibition was prevented by TMP treatment. The EC50 right-shifted from −6.04 ± 0.09 to −5.74 ± 0.09 Log M ($p < .05$) in tunicamycin-exposed arteries, and in arteries co-treated with tunicamycin and TMP the value was −6.27 ± 0.19 Log M (p < .05 vs. tunicamycin, $p > .05$ vs. control).

Arteries treated with TMP but without homocysteine or tunicamycin exposure showed no alterations in the BK_{Ca} channel-mediated relaxation (Fig. 2A & B). In both sets of experiments, no differences were observed in the resting force and the U46619-induced pre-contraction among different treatment groups (Table 1).

3.3. TMP prevents inhibition of BK_{Ca} channel current in homocysteine-exposed PCASMCs

PCASMCs exposed to homocysteine showed significantly suppressed K^+ currents, largely attributable to the inhibition of BK_{Ca} channels. As shown in Fig. 3, homocysteine inhibited both basal K^+ currents (73.38 ± 5.29 vs. 96.02 ± 4.09 pA/pF in control, $p < .05$) (Fig. 3A & B *upper panel*) and the current in response to NS1619 (128.16 ± 11.55 vs. 192.85 ± 5.59 pA/pF in control, $p < .05$) (Fig. 3C & D *upper panel*). Differentiation of BK_{Ca} component with the specific BK_{Ca} channel blocker iberiotoxin showed that after homocysteine exposure the current density of BK_{Ca} channels (iberiotoxin-inhibitable current)

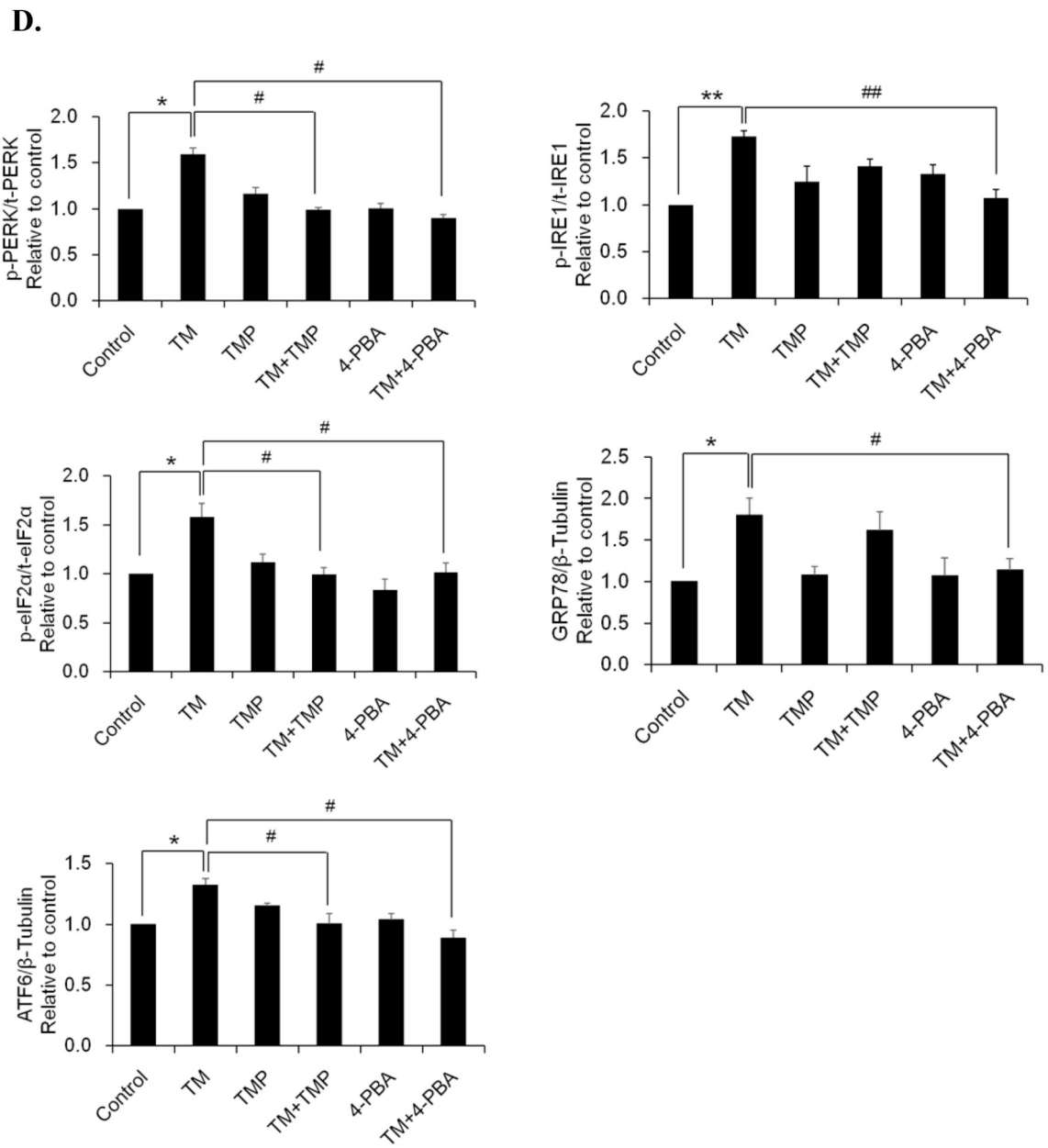

Fig. 1. (*continued*)

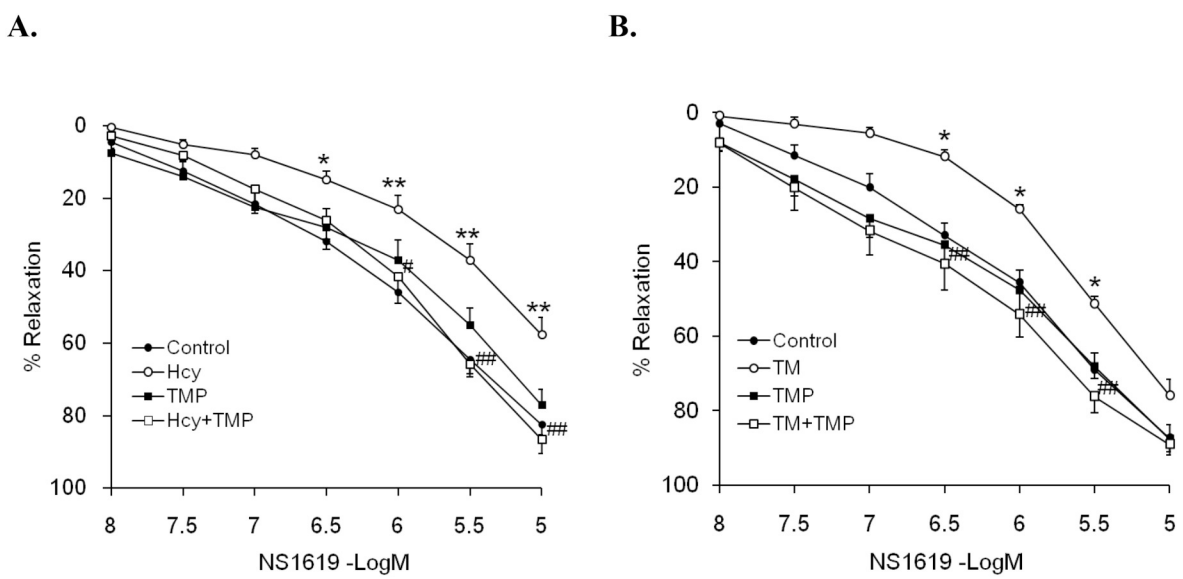

Fig. 2. TMP prevents BK_{Ca} channel dysfunction in ER-stressed porcine coronary arteries. Vasorelaxant responses of porcine small coronary arteries to the BK_{Ca} channel activator NS1619 were attenuated after exposure to homocysteine (A, $n = 8$) and tunicamycin (B, n = 8). Co-incubation of the arteries with TMP preserved the relaxant response in both homocysteine- (A) and tunicamycin- (B) exposure. N indicates the number of independent experiments. $^*p < .05$, $^{**}p < .01$ vs. control; #p < .05, ##p < .01 vs. Hcy or TM. TMP: tetramethylpyrazine, Hcy: homocysteine, TM: tunicamycin.

Table 1
Resting force and precontraction of porcine small coronary arteries subjected to different treatments.

Group	Resting force (mN)	U46619-induced precontraction (mN)
*In*Fig. 2a		
Control	2.5 ± 0.2	7.0 ± 0.7
Hcy	2.6 ± 0.5	6.3 ± 0.5
TMP	2.8 ± 0.4	10.1 ± 1.8
Hcy + TMP	2.1 ± 0.2	7.9 ± 1.0
*In*Fig. 2b		
Control	3.2 ± 0.3	8.9 ± 0.9
TM	2.6 ± 0.3	10.0 ± 1.4
TMP	3.5 ± 0.3	10.7 ± 1.6
TM + TMP	2.4 ± 0.2	9.3 ± 1.2

Data are shown as mean ± SEM. n = 8 in each group. Hcy: homocysteine, TMP: tetramethylpyrazine, TM: tunicamycin.

decreased from 63.25 ± 6.97 to 39.02 ± 5.13 pA/pF ($p < .05$) (Fig. 3A & B *lower panel*) at basal condition and from 152.33 ± 8.32 to 90.56 ± 12.47 pA/pF (p < .05) upon stimulation with the channel activator NS1619 (Fig. 3C & D *lower panel*). The inhibitory effect of homocysteine on BK_{Ca} channels was antagonized by TMP. Compared with homocysteine-exposed PCASMCs, cells exposed to homocysteine and treated with TMP exhibited significant ($p < .05$) larger current density of BK_{Ca} channels under both basal (64.93 ± 5.15 pA/pF) and NS1619-stimulated (138.11 ± 15.19 pA/pF) conditions (Fig. 3A–D *lower panel*). The enhancement of BK_{Ca} current led to an increase of the whole-cell K^+ current (95.66 ± 2.68 pA/pF at basal and 172.26 ± 18.29 pA/pF upon NS1619 activation) (p < .05 vs. homocysteine) (Fig. 3A–D *upper panel*).

3.4. TMP preserves the protein level of BK_{Ca} β1 subunit in homocysteine-exposed PCASMCs

In a recent study [29], we demonstrated that homocysteine exposure lowered the protein level of β1 subunit of BK_{Ca} in PCASMCs without significantly affecting the protein level of α subunit. Data from the present study again showed downregulation caused by homocysteine of BK_{Ca} β1 protein while not BK_{Ca} α in PCASMCs (Fig. 4A & B *left panel*). Compared to homocysteine-exposed cells, PCASMCs co-treated with homocysteine and TMP showed preserved protein level of BK_{Ca} β1 (Fig. 4B *left panel*).

The mRNA expression of either α or β1 subunit of BK_{Ca} was barely affected by homocysteine, which was consistent with our previous data [29]. TMP did not alter the mRNA expression of α and β1 subunits of BK_{Ca}, in both control and homocysteine-exposed cells (Fig. 4A & B *right panel*).

3.5. TMP inhibits homocysteine-induced BK_{Ca} β1 ubiquitination in PCASMCs

Results of ubiquitination assay (Fig. 5) showed that BK_{Ca} β1 protein from homocysteine-exposed PCASMCs more intensively interacts with the anti-ubiquitin antibody, suggesting that homocysteine significantly enhanced the ubiquitination of BK_{Ca} β1. TMP inhibited the enhancement of BK_{Ca} β1 ubiquitination caused by homocysteine. TMP itself did not affect the BK_{Ca} β1 ubiquitination level in cells without homocysteine exposure. Enhanced BK_{Ca} β1 ubiquitination was found to be concomitant with decreased BK_{Ca} β1 protein content, and suppressed BK_{Ca} β1 ubiquitination corresponding to increased BK_{Ca} β1 protein, as suggested by the blot intensity of the input cell lysate (Fig. 5).

3.6. TMP inhibits the expression of E3 ubiquitin ligases in homocysteine-exposed PCASMCs, in which inactivation of FoxO3a is involved

The expression of muscle specific E3 ubiquitin ligases atrogin-1 and Murf-1 was significantly upregulated in PCASMCs subjected to homocysteine exposure. Both the mRNA and the protein level of atrogin-1 and Murf-1 were increased. Treatment with TMP significantly inhibited homocysteine-induced upregulation of Murf-1, evidenced by a decreased mRNA and protein level of Murf-1 (Fig. 6A). In addition, TMP tended to suppress the upregulation of atrogin-1 although the inhibition was statistically insignificant (Fig. 6B). Knock down of Murf-1 prevented homocysteine-induced BK_{Ca} β1 loss in PCASMCs (Fig. 6C & D), which is an evidence supporting the role of E3 ubiquitin ligase regulation in mediating TMP-conferred BK_{Ca} β1 restoration.

In our previous study, we demonstrated that FoxO3a activation mediates the upregulation of E3 ubiquitin ligase induced by homocysteine [29]. In this study, with the finding that TMP antagonizes homocysteine on the expression of E3 ubiquitin ligases, we therefore

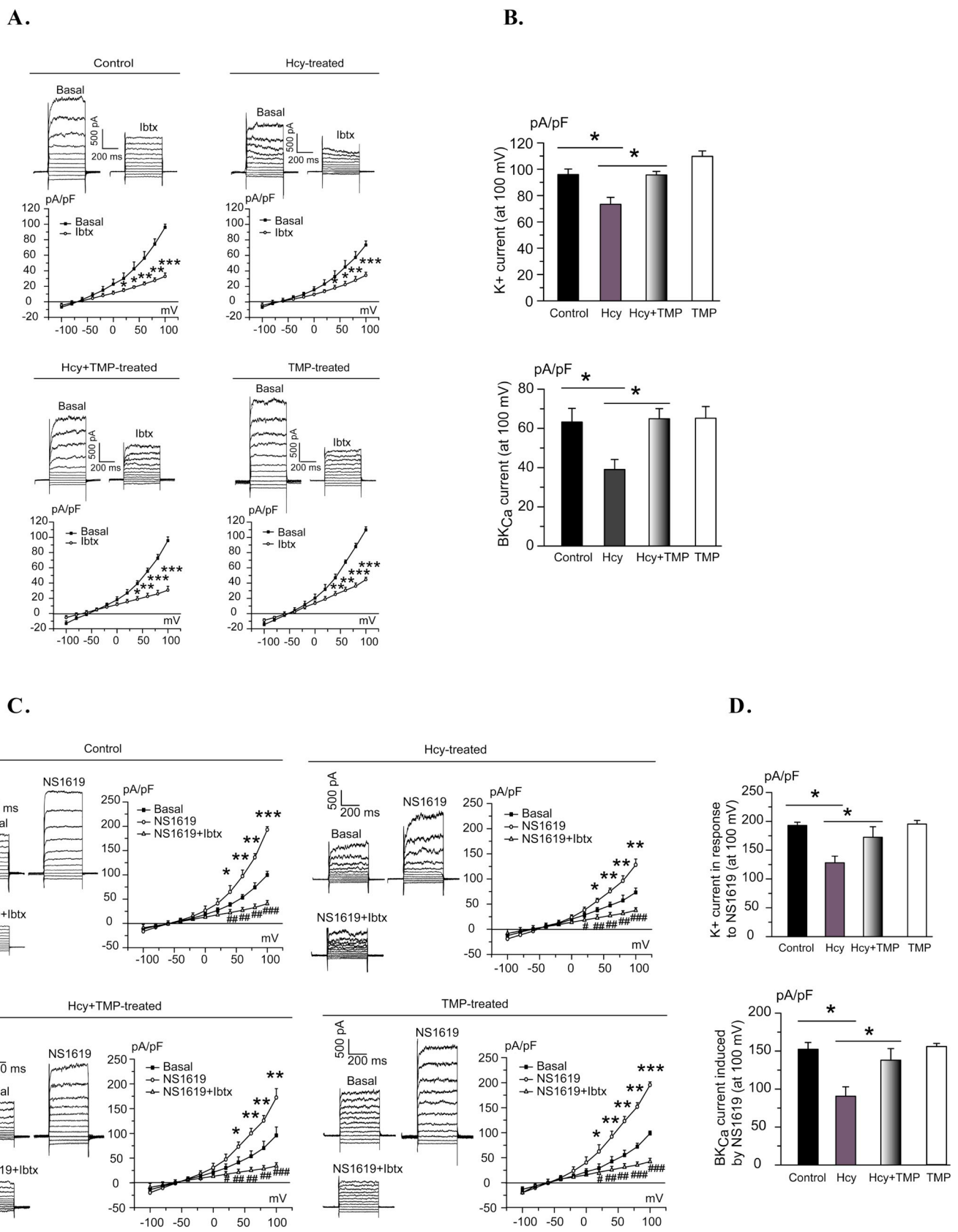

Fig. 3. TMP restores BK_{Ca} channel currents in PCASMCs whose ER is stressed by homocysteine. Original traces and I—V curves of whole-cell K^+ currents under basal condition (A) and in response to NS1619 (C) in cells from different treatment groups before and after application of the BK_{Ca} channel blocker iberiotoxin (Ibtx). *p < .05, **p < .01, ***p < .001 vs. basal, #p < .05, ##p < .01, ###p < .001 NS1619 + Ibtx vs. NS1619. Summarized data of K^+ currents and BK_{Ca} channel currents from 5 independent experiments under conditions without (B) or with NS1619 stimulation (D). TMP: tetramethylpyrazine, Hcy: homocysteine.

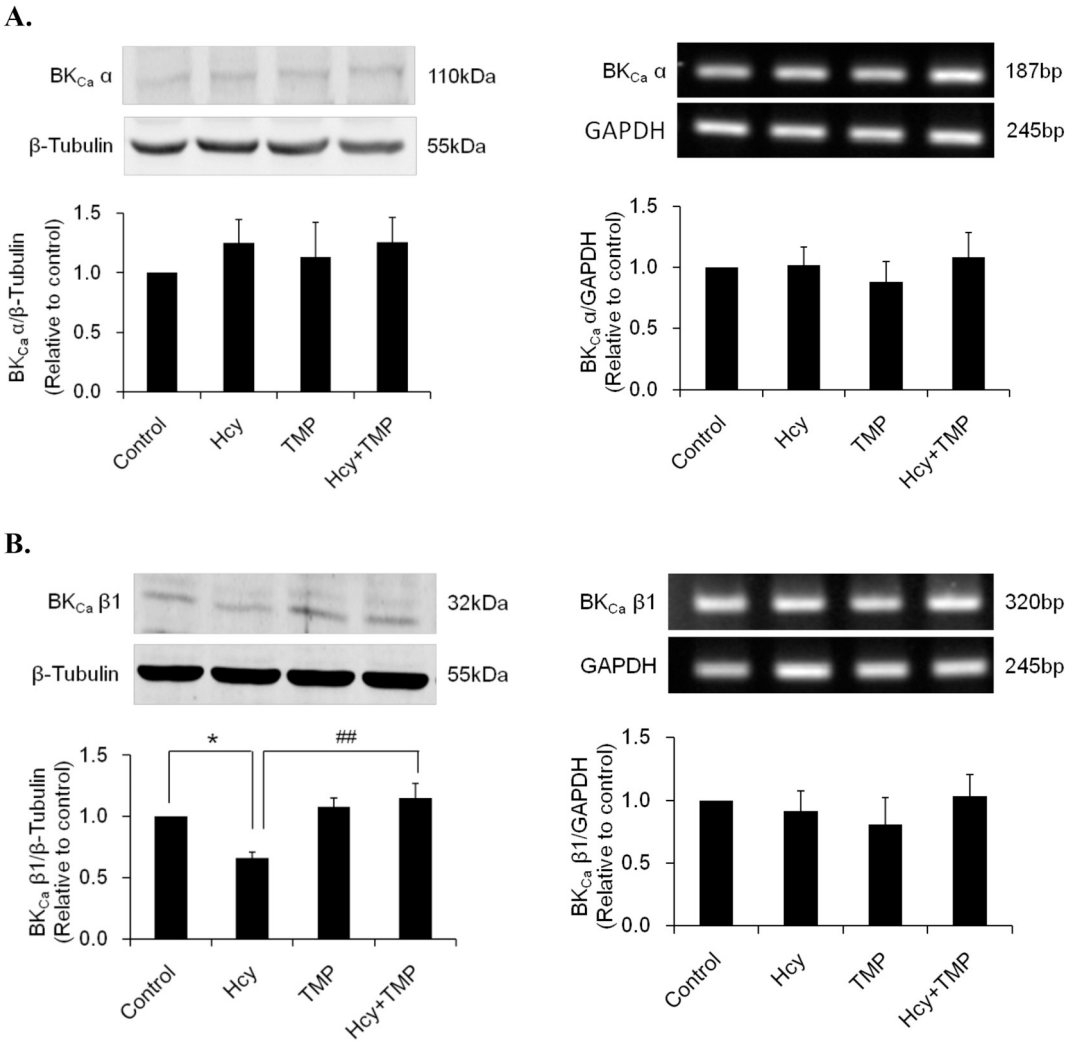

Fig. 4. TMP restores BK_{Ca} β1 protein level lowered by homocysteine in PCASMCs. Homocysteine and TMP did not alter the protein level of BK_{Ca} α subunit (A-left panel, $n = 6$) whereas had significant effect on BK_{Ca} β1 protein level (B-left panel, $n = 5$). The decrease induced by homocysteine and the restoration by TMP of BK_{Ca} β1 protein content were not due to altered transcription of BK_{Ca} β1, shown by unchanged BK_{Ca} β1 mRNA expression (B-right panel, n = 8). The mRNA expression of BK_{Ca} α was not affected by homocysteine and TMP either (A-right panel, n = 8). N indicates the number of independent experiments. *p < .05 vs. control; ##p < .01 vs. Hcy. TMP: tetramethylpyrazine, Hcy: homocysteine.

investigated the effect of TMP on FoxO3a. The results showed that TMP inhibited the translocation of FoxO3a from cytoplasm to nucleus in homocysteine-exposed PCASMCs, indicated by a decreased nuclear to cytoplasmic expression ratio of FoxO3a. As shown in Fig. 6E, without altering the overall FoxO3a protein level in PCASMCs, TMP prevented homocysteine-induced accumulation of FoxO3a in the nucleus that implies decreased FoxO3a transcriptional activity (Fig. 6F).

4. Discussion

The present study demonstrated that in porcine coronary arteries, 1) TMP protects smooth muscle BK_{Ca} channel function from homocysteine-induced impairment; 2) Prevention of the loss of β1 subunits by inhibiting ubiquitination of this subunit is involved in the protective effect of TMP against homocysteine on BK_{Ca} channel function; 3) Inhibition of ER stress-mediated FoxO3a activation and the subsequent FoxO3a-driven E3 ubiquitin ligases expression is likely responsible for the decreased BK_{Ca} β1 ubiquitination conferred by TMP.

The role of anti-inflammatory and antioxidant characteristics of TMP in conferring cardiovascular benefits has been well documented. For example, TMP suppresses the expression of pro-inflammatory mediators, enhances SOD activity, inhibits NADPH oxidase, and regulates mitochondrial biogenesis in vascular endothelial cells [12,21,31,37]. TMP-conferred cardioprotection is also attributable to its vasodilatory action that involves both endothelium-dependent, e.g. NO pathway [24,27], and -independent mechanisms, e.g. activation of K_{ATP} and SK_{Ca} channels in smooth muscle cells [15,30,35]. In this study, we observed that TMP effectively inhibits UPR in smooth muscle cells whose ER is stressed, either by homocysteine or tunicamycin. Further comparison of TMP with the classic ER stress inhibitor 4-PBA showed equivalent effectiveness in inhibiting the expression and activation of ER stress sensor molecules. Together with our latest study of coronary endothelial cells in which we for the first time reported the anti-ER stress capacity of TMP [19], the present investigation in coronary smooth muscle added further evidence in support of the significance of anti-ER stress property in vasoprotection conferred by this traditional Chinese medicine. Through inhibiting ER stress, TMP protected the vasodilatory function of smooth muscle BK_{Ca} channels, evidenced by the restored relaxant response of endothelium-denuded coronary arteries to NS1619 under exposure to tunicamycin and homocysteine.

We previously demonstrated that in porcine coronary arteries, homocysteine impairs endothelium-dependent relaxation through a mechanism involving ER stress-mediated inhibition of IK_{Ca} and SK_{Ca}

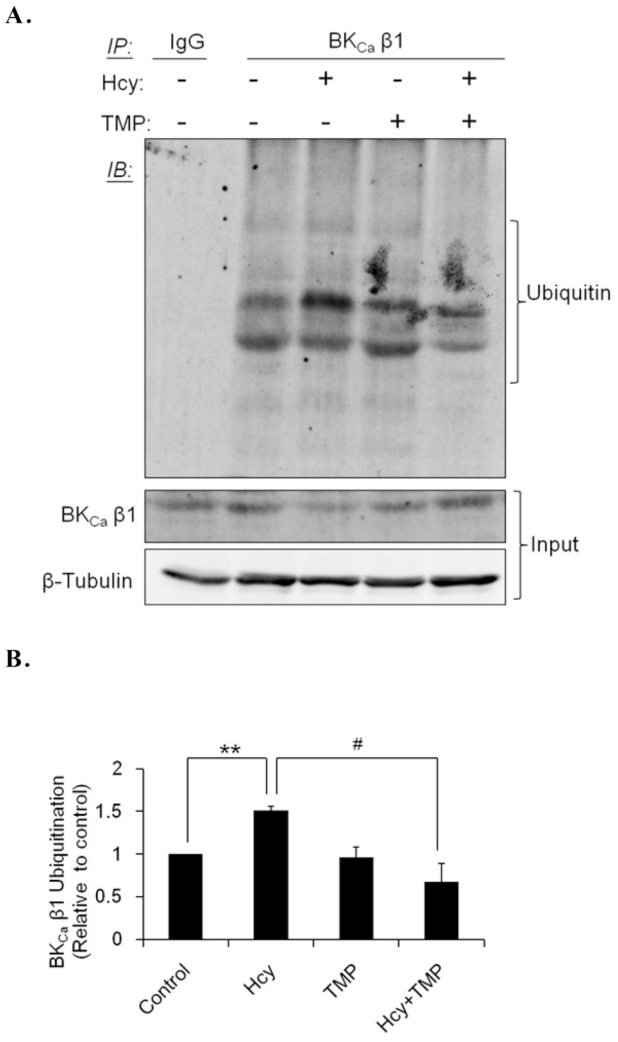

Fig. 5. TMP inhibits homocysteine-induced BK_{Ca} β1 ubiquitination. A: Representative blots of ubiquitinated BK_{Ca} β1. B: Summarized data from 4 independent experiments. Homocysteine significantly enhanced the ubiquitination of BK_{Ca} β1, which was inhibited by TMP. The level of BK_{Ca} β1 ubiquitination was reversely correlated with BK_{Ca} β1 protein content. **p < .01 vs. control, #p < .05 vs. Hcy TMP: tetramethylpyrazine, Hcy: homocysteine.

channels in endothelial cells [32]. In addition, we observed that ER stress contributes to eNOS inhibition in homocysteine-exposed coronary endothelial cells by modulating Akt and eNOS phosphorylation (unpublished data). Although the effect of TMP on coronary endothelial function especially the role of ER stress in the regulatory effect conferred by TMP was not studied under hyperhomocysteinemic conditions, given the anti-ER stress characteristic of TMP in both smooth muscle cells (current study) and endothelial cells [19] in coronary arteries, it is reasonable to postulate that TMP protects coronary dilator function from homocysteine-induced impairment by targeting on both endothelium and smooth muscle where inhibition of ER stress plays an important role. Moreover, future studies to clarify the effect of TMP on endothelial IK_{Ca} and SK_{Ca} channels and ER stress signal transduction involved in the modulation will help complete the picture of the effect of TMP on the K_{Ca} family in coronary vasculature.

In this study, coronary arteries were subjected to TMP treatment prior to the vasoactivity study and the isometric force recording was performed under the condition that TMP was washed out, the results therefore did not reflect the instant effect of TMP on BK_{Ca} channels. Though whether TMP directly acts on smooth muscle BK_{Ca} channels to dilate coronary arteries was out of the scope of the present study, it is worthy of investigation. There were studies showing failure of BK_{Ca} channel blocker in modifying TMP-induced decrease of $[Ca^{2+}]_i$ and membrane potential in cultured smooth muscle cells and in regulating TMP-induced relaxation in pre-constricted rat aorta [15,30,35], suggesting that BK_{Ca} channels are not involved in the intrinsic vasodilatory effect of TMP, which may also apply to the coronary vasculature although studies are needed to clarify this.

We further demonstrated that restoration of BK_{Ca} channel dilator function by TMP is a result of restoration of the BK_{Ca} channel activity. PCASMCs exposed to homocysteine showed a significant suppression in the BK_{Ca} channel current, which is consistent with the results presented in our previous study [29]. TMP prevented BK_{Ca} channel inhibition. In PCASMCs co-treated with homocysteine and TMP, the BK_{Ca} channel current, both basal and in response to the channel activator NS1619, was observed to be restored to the normal level. Western blot analysis related the restored BK_{Ca} channel activity to the normalized protein level of BK_{Ca} β1 subunits.

The reduced protein level while the unchanged mRNA level of BK_{Ca} β1 suggests that protein degradation might be a basis of the BK_{Ca} β1 loss. It is well known that ubiquitination of the target protein is prerequisite to its degradation by the proteasome [34]. We therefore performed ubiquitination assay and the results supported our assumption. Exposure to homocysteine enhanced BK_{Ca} β1 ubiquitination in PCASMCs while TMP reversed the enhancement caused by homocysteine. The level of BK_{Ca} β1 ubiquitination in PCASMCs co-treated with homocysteine and TMP did not differ from that of the control cells.

Further experiments aimed to unravel the molecular basis of TMP-conferred inhibition of BK_{Ca} β1 ubiquitination. In our recent study using the same vessel (porcine coronary arteries) and the same cell (PCASMCs) preparations, we demonstrated that activation of the transcription factor FoxO3a by ER stress is responsible for homocysteine-induced BK_{Ca} β1 loss and the E3 ubiqutin ligase atrogin-1 is a critical downstream target of FoxO3a in mediating the loss of BK_{Ca} β1 [29]. Considering the inhibitory effect of TMP on homocysteine-induced ER stress and its restoration of BK_{Ca} β1 level against homocysteine, it is reasonable to investigate whether modulation of FoxO3a-E3 ubiquitin ligases axis is involved in TMP-conferred preservation on BK_{Ca} β1. Our results showed that TMP prevents FoxO3a from homocysteine-induced activation, which is suggested by the decrease of nuclear localization of FoxO3a. Determination of FoxO3a-dependent muscle-specific E3 ubiquitin ligase, i.e. atrogin-1 and Murf-1 [5,25], showed that in TMP-treated PCASMCs, the decreased nuclear to cytoplasmic expression ratio of FoxO3a is associated with a significant downregulation of Murf-1 expression and a marginal reduction in atrogin-1 level. Furthermore, in homocysteine-exposed PCASMCs that transfected with Murf-1 siRNA, we observed restoration of the protein level of BK_{Ca} β1. Taken together, these experiments demonstrated that TMP prevents homocysteine-enhanced BK_{Ca} β1 protein ubiquitination thereby inhibits BK_{Ca} β1 degradation. Inhibition of ER stress-mediated FoxO3a activation and FoxO3a-dependent E3 ubiquitin ligase expression is involved in the preventive effect of TMP against homocysteine on the ubiquitination of BK_{Ca} β1.

In summary, we demonstrated that TMP protects coronary dilator function from homocysteine-induced impairment. Reversal of BK_{Ca} channel inhibition through suppressing ER stress-mediated loss of BK_{Ca} β1 subunits underlies TMP-conferred protection, in which inhibition of FoxO3a-driven E3 ubiquitin ligase expression plays an important role. Results derived from this study provide new mechanistic insights into the vasoprotective effect of TMP, which may help broadening the

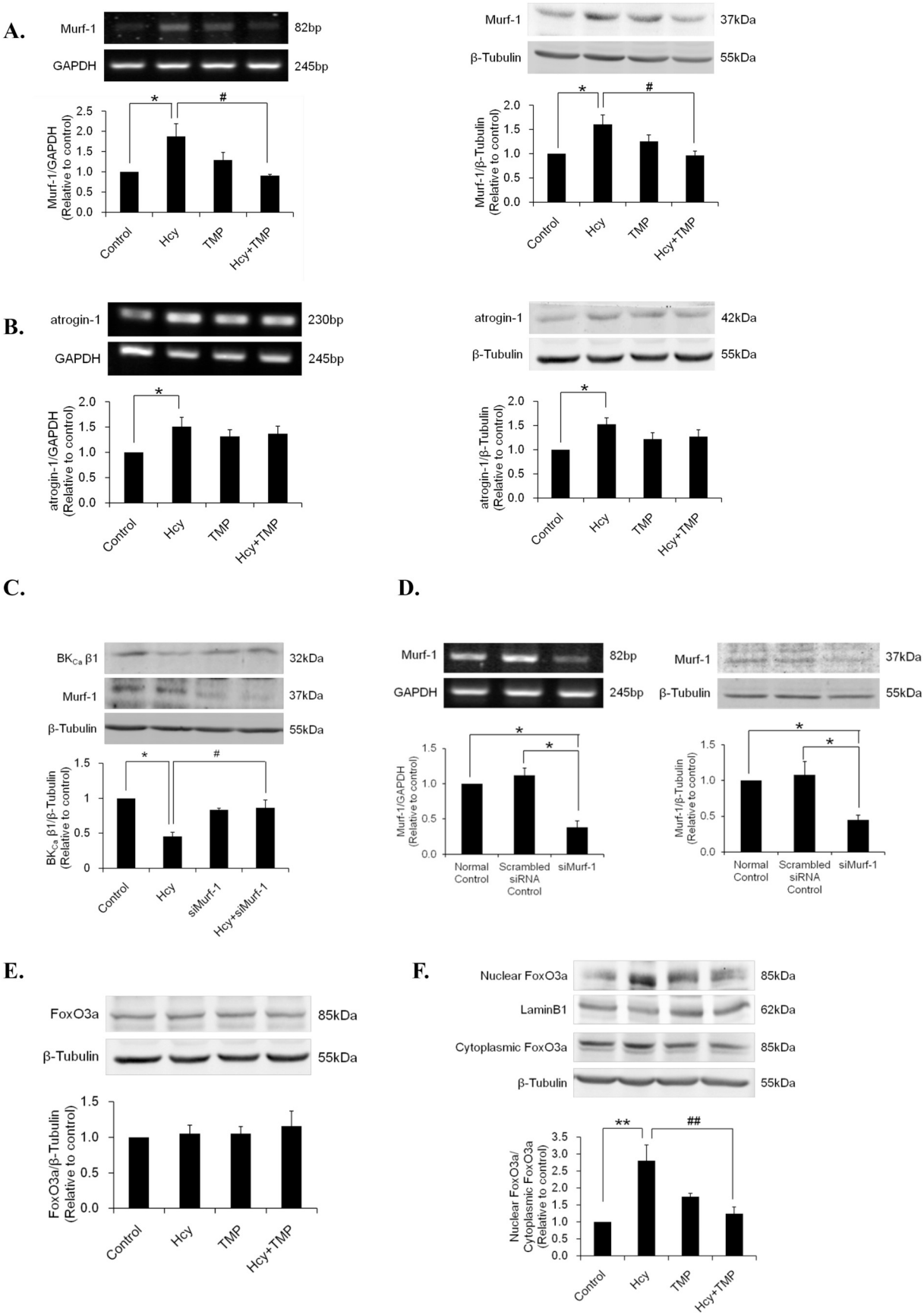

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Fig. 6. TMP inhibits homocysteine-induced expression of E3 ubiquitin ligases and nuclear translocation of FoxO3a in PCASMCs. Expressions of Murf-1 (A) and atrogin-1 (B) were increased by homocysteine at both the mRNA (A&B-left panel) and the protein level (A&B-right panel). Co-treatment of TMP significantly inhibited homocysteine-induced Murf-1 upregulation (A, $n = 9$) and tended to suppress atrogin-1 expression (B, n = 9). Knock down of Murf-1 by siRNA prevented homocysteine-induced decrease of BK_{Ca} β1 protein (C, $n = 4$). Effective knockdown was demonstrated by a significant decrease (62%) in mRNA expression of Murf-1 that resulted in 55% downregulation of Murf-1 protein (D, n = 5). While the overall level of FoxO3a expression remained unchanged (E, n = 6), nuclear to cytoplasmic expression ratio of FoxO3a was significantly increased by homocysteine, which was inhibited by TMP (F, n = 6). N indicates the number of independent experiments. *p < .05, **p < .01 vs. control; #p < .05, ##p < .01 vs. Hcy. TMP: tetramethylpyrazine, Hcy: homocysteine.

therapeutic use of this traditional Chinese medicine.

Acknowledgements

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Declarations of interest

None.

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