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Usnic acid inhibits ER stress activation through AMPK signaling pathway in rat cardiomyocytes

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Abstract. – OBJECTIVE: In the present study, we investigated the effects of usnic acid (UA) on the endoplasmic reticulum (ER) stress processes in rat cardiomyocytes.

MATERIALS AND METHODS: Gene expression of pro-inflammatory cytokines and activation of ER stress signaling were analyzed. Besides, levels of phosphorylated AMPK were measured to evaluate the mechanisms of UA. Finally, small interfering RNA (siRNA) oligos targeting AMPK subunits were used to determine the roles of AMPK in rat cardiomyocytes treated with UA.

RESULTS: We found that UA treatment significantly reduced ER stress activation and expression of pro-inflammatory cytokines, via an AMPK signaling-dependent manner.

CONCLUSIONS: UA might be useful to reduce the occurrence of adverse cardiovascular events.

Key Words:
AMPK signaling, Cardiomyocyte, Endoplasmic reticulum stress, Usnic acid.

Introduction

Endoplasmic reticulum (ER) stress occurs when the amount of unfolded protein exceeds the folding capacity of the ER[1-4]. It is activated by several signaling networks including metabolic starvation, hypoxia and pro-inflammatory cytokines[5,6]. Until now, it has been well known that three ER-resident transmembrane proteins are activated to initiate and mediate ER stress: activating transcription factor 6 (ATF6), inositol-requiring enzyme 1 (IRE1) and pancreatic ER kinase-like ER kinase (PERK)[7,8].

Increasing evidence suggests that ER stress plays critical roles in the physiological and pathophysiological condition, including obesity, tumorigenesis and ageing[9,10]. Besides, recent studies indicate that ER stress might be a candidate instigator for pathological cell death and functional change intimately involved in the maintenance of vascular and cardiac health[11-13]. In addition, ER stress-induced cell apoptosis was identified as a key cause for the pathogenesis of ischemic heart diseases and heart failure[11,13].

Usnic acid (UA) is a naturally occurring dibenzofuran derivative found in several lichen species, which has exhibited anti-proliferative roles in tumor cells. However, its effect on ER stress in cardiomyocytes has not yet been investigated. Therefore, we wanted to study whether UA is involved in ER stress induction in the heart, and, more specifically, which these pathways are regulated by UA. Using a rat cardiomyocyte system, we found that UA differentially induces ER stress pathways. These results might be important in understanding the effect of UA on the heart, but they also shed light on the possible mode of action of the drug development.

Materials and Methods

Cell Culture and Ragents

Rat cardiomyocytes were isolated and cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 g/ml streptomycin. Usnic acid, tunicamycin and vehicle control (DMSO) were purchased from Sigma-Aldrich Company (Saint Louis, MO, USA).

siRNA, RNA Extraction and Real-time Analysis

siRNA oligos targeting AMPKa1 and AMPKa2 were purchased from Genepharma (Shanghai, China). A scramble siRNA was used as a negative control. Total RNA was isolated using

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the TRIzoL reagent (Invitrogen, Carlsbad, CA, USA). Reverse transcription was conducted from 1 μg total RNA using Oligo-dT primers and M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Real-time PCR (polymerase chain reaction) assays were performed on a Mini-iOpticonTM Real-Time PCR system (BioRad, Hercules, CA, USA). Thermal cycling conditions were as follows: activation of DNA polymerase at 95°C for 5 min followed by 45 cycles of amplification at 95°C for 10 s and at 60°C for 45 s. mRNA expression of β-actin gene was used as an internal control.

Western Blot

Cells were harvested and lysed with lysis buffer [(50 mM Tris-HCl, pH 7.4, 100 mM DTT (dithiothreitol), 2% w/v SDS (sodium dodecyl sulphate), 10% glycerol)]. After centrifugation at 20.000 g for 10 min at 4°C, proteins in the supernatants were quantified and separated by 12% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel electrophoresis), transferred to PVDF (polyvinyldene fluoride) membranes. After blocking with 5% nonfat milk, membranes were immunoblotted with indicated antibodies, followed by HRP (horse-radish peroxidase)-linked secondary antibodies. The signals were detected by Millipore SuperSignal®, Rockford, IL, USA HRP Substrate kit according to manufacturer's instructions. Antibodies were purchased from Cell Signaling Company and Abcam Company (Cambridge, MA, USA).

Statistical Analysis

Data were analyzed with the PRISM 5.0 software package (GraphPad, San Diego, CA, USA). Results are expressed as the mean S.E. and were compared by Student's *t* test or analysis of variance. A value of $p < 0.05$ was considered significant.

Results

Usnic Acid Prevents ER Stress Activation in Rat Cardiomyocytes

Freshly isolated and cultivated neonatal rat cardiomyocytes provide a unique cell system to study drug effects on heart cells. To analyze the effect of usnic acid (UA) on cardiomyocytes, we treated freshly isolated neonatal rat cardiomyocytes with 2 mM UA for 48 hours. As shown in Figure 1A, tunicamycin (TM), a classical ER stress inducer, activated phosphorylated PERK (protein kinase-like endoplasmic reticulum kinase) and eIF2a, two markers of ER stress activation. However, UA treatment significantly reduced their protein levels, suggesting an alleviation of ER stress (Figure 1A). Besides, message RNA levels of ATF4, EMDM and CHOP, down-stream target genes of ER stress, were also down-regulated by UA treatment (Figure 1B-D). The inhibition of CHOP (C/EB homologous protein) Pwas also confirmed by western blot, indicating that UA could repress ER stress-induced cell apoptosis (Figure 1E).

Usnic Acid Reduces Expression of Pro-inflammatory Cytokines in Rat Cardiomyocytes

Activation of ER stress usually results in inflammatory responses[14]. Therefore, we speculate that whether UA may down-regulate expression levels of pro-inflammatory cytokines. As expected, UA treatment markedly inhibited the mRNA levels of IL-1β and TNF-α, which were up-regulated by tunicamycin (Figure 2A-B). Besides, the down-regulation of IL-1β and TNF-α was also confirmed by ELISA-based protein quantification (Figure 2C-D).

Usnic Acid Prevents ER Stress Activation in vivo

Next, the roles of UA in ER stress were examined *in vivo*. Rats were divided into two groups, which were treated with tunicamycin and tunicamycin plus UA, respectively. In agreement, we observed a reduced ER stress activation in rats treated with UA, as shown by phosphorylated PERK and eIF2α (Figure 3A). Moreover, the down-regulation of ATF4 (activating transcription factor4), EMDM (Emery-Dreyfuss muscular-distrophy protein) and CHOP was also observed (Figure 3B-E), suggesting that UA also prevented ER stress activation *in vivo*.

Usnic Acid Activates AMP Kinase Signaling

Next, we tried to seek the molecular mechanisms of inhibition of ER stress by UA. As shown in Figure 4, treatment of rat cardiomyocytes with UA activated the phosphorylation of AMP kinase (5' AMP-activated protein kinase) (Figure 4). Consistently, phosphorylated ACC (acetyl-CoA carboxylase), a down-stream target of AMP kinase, was also activated by UA (Figure 4). However, phosphorylated ERK1/2, P38 and AKT were also changed by UA treatment (Data not shown).

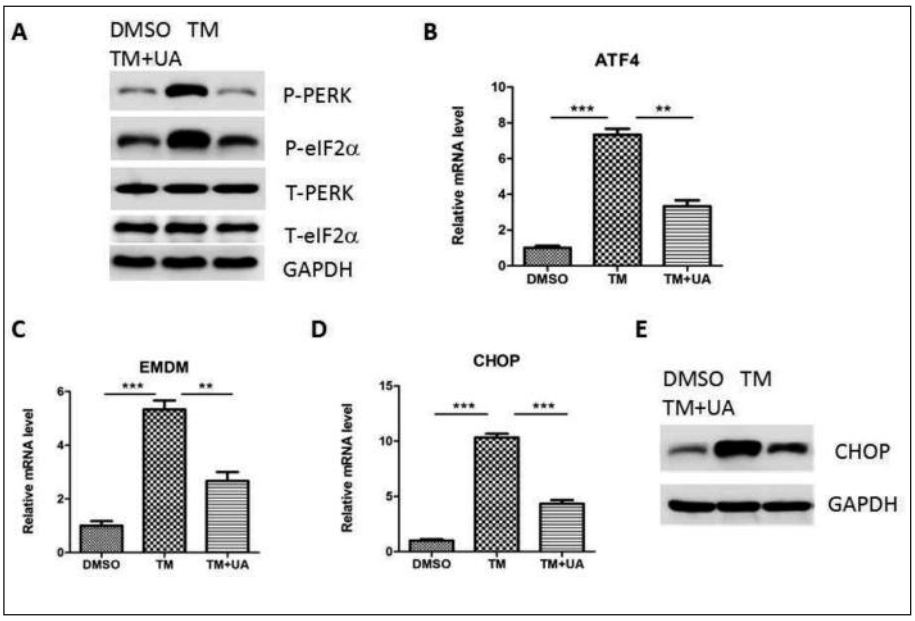

Figure 1. Usnic Acid prevents ER stress activation in rat cardiomyocytes. **A**, Representative protein levels of phosphorylated PERK and eIF2a in rat cardiomyocytes treated with vehicle control (DMSO: dimethyl sulfoxide), tunicamycin (TM) or tunicamycin plus Usnic Acid (UA). **B-D**, mRNA levels of ATF4, EMDM and CHOP were determined by real-time PCR analysis in rat cardiomyocytes. E, Representative protein levels of CHOP in rat cardiomyocytes.

Knockdown of AMPKα1 and AMPKα2 Subunits Abolished Roles of UA

We next test whether AMPK signaling is required for the inhibiting effect of UA on ER stress activation in rat cardiomyocytes. As shown in Figure 5A and 5B, cells were infected with small interfering RNA targeting AMPKα1 and AMPKα2 subunits, which significantly inhibit endogenous AMPKα1 and AMPKα2 expression (Figure 5A-B). The ablation of endogenous AMPK signaling significantly inhibited the ability of UA to expression levels of ER stress mark-

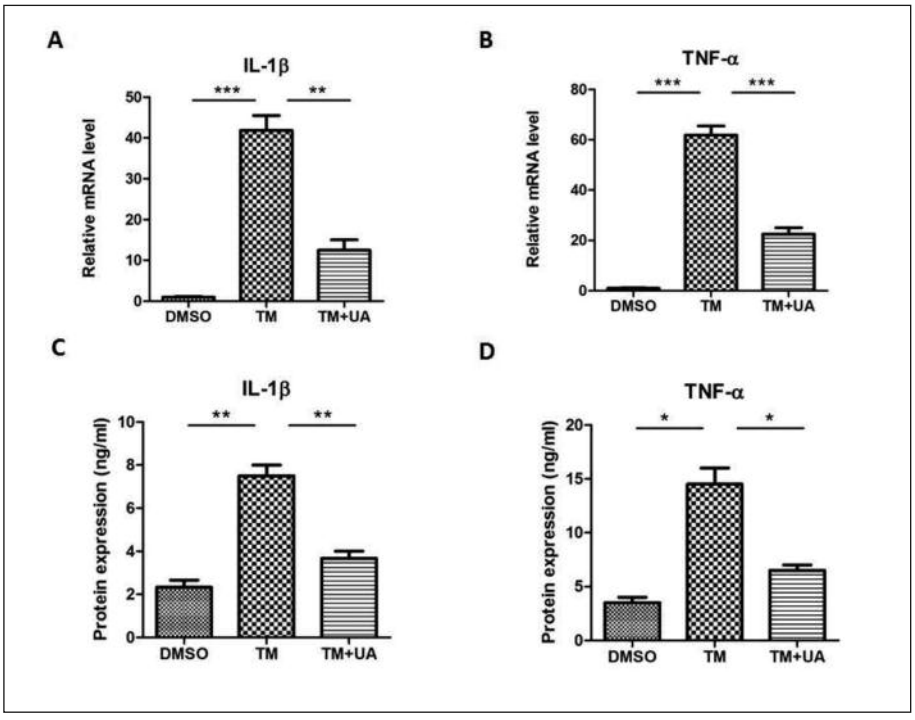

Figure 2. Usnic Acid reduces expression of pro-inflammatory cytokines in rat cardiomyocytes. **A-B**, Relative mRNA expression of TNFa and IL-1β was assessed by Real-time PCR in rat cardiomyocytes treated as indicated. C-D, Determination by ELISA of TNF-α and IL-1β secretion into the culture media.

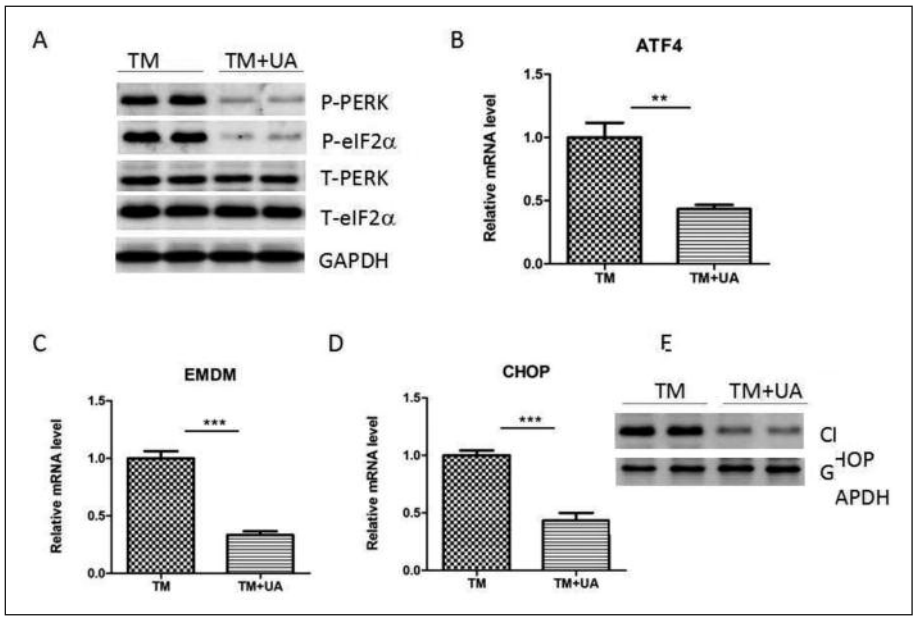

Figure 3. Usnic Acid prevents ER stress activation *in vivo*. **A**, Representative protein levels of phosphorylated PERK and eIF2a in rat hearts treated with vehicle control (DMSO), tunicamycin (TM) or tunicamycin plus Usnic Acid (UA). **B-D**, mRNA levels of ATF4, EMDM and CHOP were determined by real-time PCR analysis in rat hearts. E, Representative protein levels of CHOP in rat hearts.

ers (Figure 5C), demonstrating that AMPK signaling plays an indispensable role in UA-inhibited ER stress activation.

Discussion

Following myocardial infarction, quiescent cardiac fibroblasts are transformed into a proliferative and invasive myofibroblast phenotype, which accounts for a major source of cytokines including TNF-α, IL-1β and TGF-β, leading to the activation of ER stress pathway. Persistent ER stress activation promotes remodeling and apoptosis in cardiomyocytes[15]. Besides, it contributes to decreased cardiac function, and faster progression to heart failure and cardiac hypertrophy.

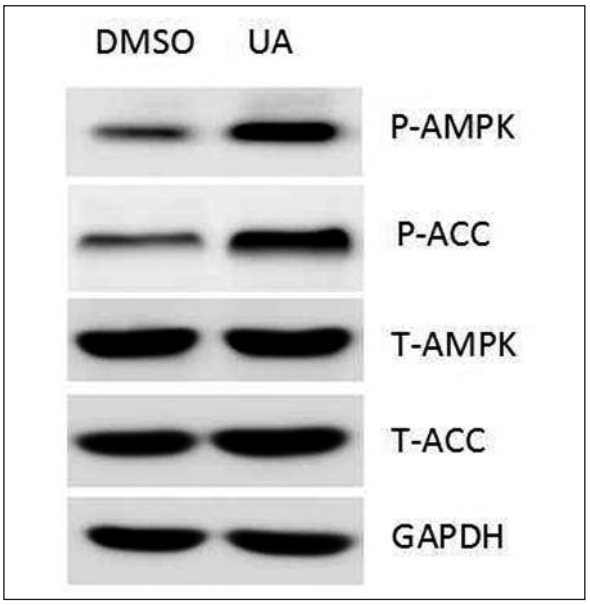

Figure 4. Usnic Acid activates AMP kinase signaling. Phosphorylation of AMPK and ACC was analyzed in rat cardiomyocytes treated with UA or vehicle control (DMSO).

In the present study, we demonstrated that Usnic Acid reduced ER stress activation in rat cardiac cells, in a process that coincides with the activation of AMPK signaling. Therefore, activation of the AMPK pathway by Usnic Acid might be involved in the cardioprotective effects of this compound and, since it may improve cardiac performance, it might be useful to reduce the occurrence of adverse cardiovascular events. UA has been shown some biological and physiological activities, for its active compound mainly found in lichens[16,17]. For instance, Usnic acid could inhibit breast tumor angiogenesis and growth by suppressing VEGFR2-mediated AKT and ERK1/2 signaling pathways[18]. However, the change of AKT and ERK1/2 signaling was not observed in rat cardiomyocytes treated with UA, suggesting its cell or tissue-specific roles. Indeed, we found that AMPK signaling was activated by UA treatment, as evidenced by enhanced phosphorylated AMPK and ACC. Previous studies have shown that activation of AMPK could inhibit glucose or palmitate-induced ER stress in hepatocytes and pancreatic islets[19,20]. Moreover, isoproterenol instigates cardiomyocyte apoptosis and heart failure via AMPK in-

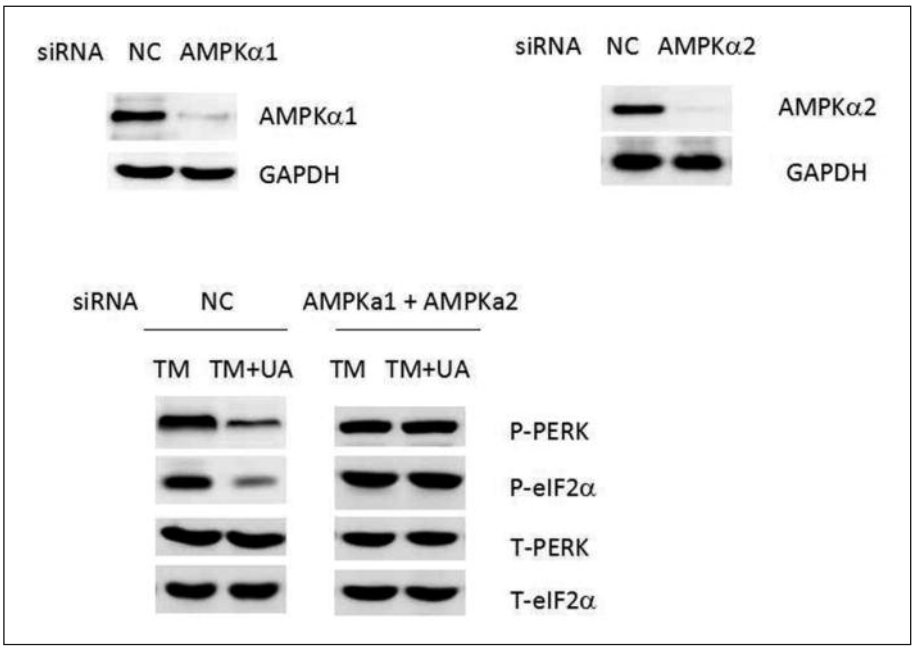

Figure 5. Knockdown of AMPKa1 and AMPKa2 subunits abolished roles of UA. **A-B**, Representative protein levels of AMPKa1 and AMPKa2 in rat cardiomyocytes transfected with siRNA oligos targeting AMPKa1, AMPKa2, or negative controls (NC). **C**, Representative protein levels of phosphorylated PERK and eIF2a in rat cardiomyocytes treated with tunicamycin (TM) or tunicamycin plus Usnic Acid (UA). Cells were pre-transfected with siRNA oligos for 20 hr.

activation-mediated endoplasmic reticulum stress[21]. Therefore, together with other studies, our results suggest that AMPK signaling activator might be a promising cardiac cell protector *in vivo* and *in vitro*.

Conclusions

Our results, for the first time, uncovered the cardioprotective roles of Usnic Acid, which may be useful to reduce the occurrence of adverse cardiovascular events.

Acknowledgements

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Conflict of interest

The authors declare that there are no conflicts of interest.

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