Herpud1 suppress angiotensin II induced hypertrophy in cardiomyocytes

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ABSTRACT

Purpose: The purpose of this study was to analyze the role of homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 (Herpud1) gene in the development of cardiomyocyte hypertrophy.

Method: In order to examine the effect of suppressing Herpud1 expression, Herpud1 small interfering RNA (siRNA) was introduced into H9C2 cells, which are cell lines derived from rat myocardium, and the degree of Herpud1 protein expression and cell hypertrophy in the Herpud1 siRNA-transfected group and the control group was compared by immunostaining 48 h after Herrpud1 siRNA introduction. To examine whether hypertrophy induced by angiotensin II (Ang II) can be suppressed by the overexpression of Herpud1, the green fluorescent protein (GFP)-Herpud1 plasmid was introduced into H9C2 cells, and the degree of cell hypertrophy was examined in the GFP-Herpud1-and control groups for 48 h. Nuclear translocation of nuclear factor of activated T-cells, cytoplasmic 4 (NFATc4), a transcription factor for hypertrophic genes, was also examined.

Results: [1] Herpud1 siRNA-transfected cells showed decreased Herpud1 protein expression and hypertrophy formation compared to control cells [2]; Overexpression of Herpud1 suppresses Ang II-induced cell hypertrophy; and [3] Overexpression of Herpud1 inhibits nuclear translocation of NFATc4.

Discussion: It was suggested that Herpud1 might be an anti-hypertrophic gene in Ang II induced cardiomyocytes hypertrophy.

1. Introduction

Homocysteine-responsive endoplasmic reticulum-resident ubiquitin-like domain member 1 (Herpud1) is thought to play a role in both the unfolded protein response (UPR) and the ER-associated protein degradation (ERAD) system [1–3]. Recently, one study has shown the role of Herpud1 in cardiac function. Torrealba et al. reported that Herpud1 KO mice showed cardiac hypertrophy with a larger heart weight and thickness of the left ventricular (LV) wall. The cross-sectional area of cardiomyocytes also increased [4]. They also reported that Herpud1 small interfering RNA (siRNA)-treated neonatal rat ventricular myocytes (NRVMs) showed increased inositol 1,4,5-trisphosphate (IP3) receptors (IP3Rs) and increased cytosolic Ca2+ levels. However, the effect of overexpression of Herpud 1 on cardiomyocytes remains unknown.

In our previous study, we reported that RyR2 V3599K KI mice, in which the calmodulin (CaM) binding domain of the myocardial ryanodine receptor (RyR2) was mutated to enhance the affinity of CaM to RyR2 [5], do not develop cardiac hypertrophy in the transverse aortic coarctation (TAC) model [6]. In this model, LV systolic and diastolic function is maintained without LV hypertrophy, even when the LV peak pressure is increased in WT mice. RNA-seq analysis of these mice revealed that Herpud1 was more expressed in RyR2 V3599K mice than in WT mice only in the TAC models. Taken together, these findings suggest that overexpression of Herpud1 may suppress cardiac hypertrophy.

The purpose of this study was to investigate, using H9C2 cells, a clonal cell line from the rat heart [7], to determine the effect of Herpud1 in cardiac hypertrophy.

In this study we employed Angiotensin II (Ang II) as a hypertrophy inducer. Ang II is associated with the pathogenesis of cardiac hypertrophy and heart failure [8]. To mimic TAC pathogenesis in vitro, we thought Ang II is the best inducer of hypertrophy.

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2. Materials and methods

2.1. Chemicals

High-glucose Dulbecco’s Modified Eagle Medium (DMEM) with L-glutamine, phenol red, sodium pyruvate, and penicillin-streptomycin solution was purchased from Wako (Japan). Fetal bovine serum (FBS) of South American Origin was purchased from Corning (NY, USA). Herpud1 Silencer Pre-designed siRNA was purchased from Ambion Thermo Fisher Scientific (Waltham, MA, USA). Herpud1 plasmid was purchased from OriGene (MD, USA). Ang II was purchased from Wako (Japan).

2.2. Antibodies

Herpud1 polyclonal antibody (PA5-59485) was purchased from Invitrogen and used at a 1:2500 dilution for IF and WB. Anti-nuclear factor of activated T-cells, cytoplasmic 4 (NFATc4) antibody (ab3447) was purchased from Abcam and used at a 1:100 dilution for IF and 1:500 for WB. Anti-GAPDH antibody (ab8245) was purchased from Abcam and used at a 1:1000 dilution for WB. Anti-Lamin antibody (ab108595) was purchased from Abcam and used at a 1:1000 dilution for WB. The secondary antibodies Alexa Fluor® 488 goat anti-rabbit immunoglobulin G (IgG) (A11008) and Alexa Fluor® 633 goat anti-rabbit IgG (A21207) were purchased from Invitrogen and used at a 1:300 dilution. Alexa Fluor® 555 goat anti-rabbit IgG (ab150078) was purchased from Abcam and used at a 1:300 dilution.

2.3. H9C2 cell culture

The H9C2 cell line was obtained from KAC Co., Ltd., Japan (Cat. No. EC88092904-F0) and cultured in high-glucose DMEM with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO2. Cells were fed every 2–3 d and subcultured when they reached 80% confluence to prevent the loss of differentiation potential.

2.4. Silencing Herpud1

After 16 h of starvation with 0% FBS, 25 nM siRNA was transfected into H9C2 cells using TransIT-TKO (Mirus Bio, WI, USA) and incubated for 48 h in 0.1% FBS. The cells were then used for immunofluorescence staining. For western blot analysis, cells were washed with PBS three times and cell lysate was collected using RIPA Lysis buffer (ATTO, Tokyo, Japan).

2.5. Overexpression of Herpud1

H9C2 cells were transiently transfected 2 ng/μL of Herpud1-enhanced green fluorescent protein (EGFP) plasmid DNA or EGFP plasmid using GenomONE GX001 (Cosmo Bio, Japan) and incubated with 0% FBS. A total of 4 h following transfection, 1 μM Ang II was added to the medium and incubated for 48 h in 0.1% FBS. The cells were used for the immunofluorescence staining. For western blot analysis, cells were washed with PBS three times and cell lysate was collected using RIPA Lysis buffer (ATTO, Tokyo, Japan).

2.6. NFATc4 translocation

H9C2 cells were transfected with 2 ng/μL Herpud1-EGFP plasmid DNA, or EGFP plasmid using GenomONE GX001, or 25 nM siRNA using TransIT-TKO incubated for 24 h in 0.1% FBS. To see the translocation of NFATc4, a total of 3 h following treatment with 1 μM Ang II or siRNA, the cells were fixed. For western blot analysis, cells were washed with PBS three times and cell lysate was collected using RIPA Lysis buffer (ATTO, Tokyo, Japan). Nuclear/Cytosolic Fractionation Kit (Cell Biolabs, Inc. San Diego, CA) was used to obtain the nuclear fraction and cytosolic fraction.

2.7. Immunofluorescence staining

The cultured H9C2 cells were fixed with 4% paraformaldehyde for 5 min. The cells were then permeabilized with 0.1% Triton-X for 20 min. Blocking was performed with 1% BSA and a protein block (Dako, DK) for 60 min. The cells were incubated overnight at 4 °C with an anti-Herpud1 antibody (Invitrogen Thermo Fisher Scientific, MA, USA) and an anti-NFATc4 antibody (Abcam, UK), followed by labelling with an Alexa Fluor® conjugated secondary antibody. Nuclei were stained with DAPI solution (Dojindo, Japan). Actin was co-stained with TRITC-phalloidin (Vector Laboratories, CA, USA).

2.8. Analysis of stained cells

Cell size measurements were performed as follows. Using the TRITC-phalloidin stained image, the outline of each cell was manually traced on a pen display and the cell area was measured using Image J software (ver. 1.53). The mean fluorescence intensity of immunofluorescent images was calculated using ImageJ software. In cases where the nuclei and cytosol intensity were evaluated separately, a nucleus ROI was made using DAPI image.

2.9. Western blotting

Whole cell lysate, nuclear fraction, and cytosolic fraction were de-natured in SDS-PAGE sample buffer. SDS-PAGE, blotting, and antibody detections were performed. Ten μg samples were put in each lane of 10% polyacrylamide precast gel (Biorad, Hercules, CA). After SDS-PAGE, proteins were transferred to the PVDF membrane. Goat anti-Mouse IgG with HRP (31430, Thermo Fisher, Waltham, MA) or Goat anti-Rabbit IgG with HRP (31460, Thermo Fisher, Waltham, MA) were used for secondary antibody with 1:2000 dilution. SupernSigna West Femto (34094, Thermo Fisher, Waltham, MA) were used for chemiluminescence.

2.10. Statistical analysis

One-way analysis of variance (ANOVA) with a post-hoc Tukey’s test was used for the statistical comparison of the four groups with normal distribution. The Kruskal-Wallis (KW) test with a post-hoc Dunnett’s test was used for the statistical comparison of the four groups without a normal distribution.

Pearson correlation analysis was used for the correlation analysis. A p-value less than 0.05 thought to be statistically significant.

3. Results

3.1. Silencing Herpud1 resulted in cardiac hypertrophy

As shown in Fig. 1, silencing Herpud1 gene expression using siRNA resulted in hypertrophy in H9C2 cells. The cell area was approximately 1.55 times larger than that of the transfection reagent only control. Knockdown of Herpud1 was confirmed by immunofluorescent staining revealing suppressed expression of Herpud1 protein (Fig. 1A and B). Fig. 1B shows the summarized data of cell size and Herpud1 expression level. A two-dimensional plot of cell size and H9C2 expression showed a clear negative relationship, indicating that H9C2 expression was suppressed, resulting in cell enlargement (Fig. 1C). Western blot analysis of whole cell lysate confirms the Herpud1 knockdown (Fig. 1D and E).
3.2. Overexpression of Herpud1 suppressed Ang II-induced cardiac hypertrophy

Fig. 2 depicts the results of the Herpud1 overexpression experiment. As shown in Fig. 2A, Ang II produced 1.39 times cell enlargement; however, overexpression of Herpud1 diminished Ang II-induced hypertrophy of the cells. The control GFP plasmid showed no effect. Western blot analysis of whole cell lysate confirms the Herpud1 overexpression (Fig. 2C and D).

3.3. Nuclear translocation of NFAT was suppressed by overexpression of Herpud1

The effect of Herpud1 silencing and overexpression on the nuclear translocation of NFATc4 is shown in Fig. 3. The nuclear/cytoplasmic ratio of NFATc4 was increased by Ang II; however, overexpression of Herpud1 completely blocked this translocation and suppressed cell hypertrophy. Interestingly, silencing Herpud1 also increased the nuclear/cytoplasmic ratio of NFATc4. Herpud1 might act as a blocker of NFATc4 nuclear translocation (Fig. 4). In this experiment, cells were fixed 3 h after Ang II loading, so that the cell size increase induced by Ang II was not observed. Western blot analysis of cytosolic fraction and nuclear fraction confirms the nuclear translocation of NFATc4 induced by Ang II, blocked by Herpud1 overexpression (Fig. 3C and D).

4. Discussion

The present study revealed that Herpud1 overexpression prevents cardiac hypertrophy induced by Ang II by inhibiting the nuclear translocation of NFATc4. On the other hand, silencing Herpud1 causes cardiac hypertrophy by accelerating nuclear translocation of NFATc4. This data suggests that Herpud1 might be an endogenous suppressor of cardiac hypertrophy.

The Herp family was first identified as ER stress-responsive proteins in human vascular endothelial cells [9]. Herp family expression is strongly upregulated by ER stress. Unlike the molecular chaperone...
GRP78 inside the ER, Herpud1 has a structure that protrudes from the ER membrane into the cytosol [10]. The UBL domain exists at the N-terminus of Herpud1 [10]. Herpud1 is thought to be involved in ER-associated protein degradation, which is the main mechanism of misfolding protein degradation [11].

4.1. Herpud1 and cardiac hypertrophy

Torrealba et al. reported that Herpud1 KO mice presented cardiac hypertrophy [4]. According to their data, cardiomyocytes from Herpud1 KO mouse and siHerpud1-treated NRVMs showed higher IP3R protein levels. However, the mRNA levels of IP3R did not change. They concluded that Herpud1 may have a role in the degradation of IP3R and that Herpud1 silencing suppresses the degradation speed of IP3R, resulting prevention of cytoplasmic Ca\(^{2+}\) overload.

Belal et al. [12] showed that Harpud1 interacted with Ca\(^{2+}\)-release proteins such as IP3R and RyR. Belal’s report is consistent with Torrealba’s hypothesis that Herpud1 is related to the degradation of Ca\(^{2+}\)-releasing channels.

4.2. NFAT translocation and calcineurin

The calcineurin-NFAT pathway is one of the two major Ca\(^{2+}\)-activated hypertrophy signaling pathways in cardiomyocytes. Calcineurin dephosphorylates NFAT, which leads to the nuclear translocation of NFAT. In the nucleus, NFAT acts in conjunction with the transcription factor GATA4 [13, 14]. The trigger of NFAT translocation is the dephosphorylation of NFAT by Ca\(^{2+}\)-calmodulin-activated calcineurin. Activation of the degradation of Ca\(^{2+}\)-releasing channels may cause decreased cytosolic Ca\(^{2+}\) levels. This decreased cytosolic calcium is a possible mechanism for the inhibition of calcineurin by Herpud1. Increased Ca\(^{2+}\) levels in Herpud1 silencing in skeletal muscle, as reported by Navarro-Marquez et al. [15], supports this idea. However, further studies on the mechanism of inhibition of NFAT translocation by Herpud1 are needed. In addition, other inhibition mechanisms of NFAT nuclear translocation may involve the anti-hypertrophic mechanism of Herpud1.

5. Conclusions

Overexpression of Herpud1 suppresses Ang II induced cardiac hypertrophy by inhibiting nuclear translocation of NFAT. Herpud1 may be a novel target for Ang II induced cardiac hypertrophy treatment.
6. Limitations

Our data were obtained using the H9C2 cell line, but not from real cardiomyocytes. Although H9C2 is quite similar to cardiomyocytes, our study. These should be examined further.

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Declaration of competing interest

All authors have no conflict of interest in this study.

Data availability

The data that support the findings of this study are available from the corresponding author, [T.Y.], upon reasonable request.

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