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# The Different Expression Patterns of HSP22, a Late Embryogenesis Abundant-like Protein, in Hypertrophic H9C2 Cells Induced by NaCl and Angiotensin Ⅱ

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**Background**: High-NaCl diet is a contributing factor for cardiac hypertrophy. The role of HSP22 as a protective protein during cardiac hypertrophy due to hypernatremia is unclear. Accordingly, this study aimed to establish a cellular hypernatremic H9C2 model and to compare the expression of HSP22 in $Ca^{2+}$ homeostasis between a high-NaCl and angiotensin II-induced hypertrophic cellular H9C2 model.
**Methods:** Real-time PCR was performed to compare the mRNA expression. Flow cytometry and confocal microscopy were used to analyze the cells.
**Results:** The addition of 30 mM NaCl for 48 h was the most effective condition for the induction of hypertrophic H9C2 cells (termed the *in vitro* hypernatremic model). Cardiac cellular hypertrophy was induced with 30 mM NaCl and 1 μM angiotensin II for 48 h, without causing abnormal morphological changes or cytotoxicity of the culture conditions. HSP22 contains a similar domain to that found in the consensus sequences of the late embryogenesis abundant protein group 3 from *Artemia*. The expression of HSP22 gradually decreased in the *in vitro* hypernatremic model. In contrast to the *in vitro* hypernatremic model, HSP22 increased after exposure to angiotensin II for 48 h. Intracellular $Ca^{2+}$ decreased in the angiotensin II model and further decreased in the *in vitro* hypernatremic model. Impaired intracellular $Ca^{2+}$ homeostasis was more evident in the *in vitro* hypernatremic model.
**Conclusion**: The results showed that NaCl significantly decreased HSP22. Decreased HSP22, due to the hypernatremic condition, affected the $Ca^{2+}$ homeostasis in the H9C2 cells. Therefore, hypernatremia induces cellular hypertrophy via impaired $Ca^{2+}$ homeostasis. The additional mechanisms of HSP22 need to be explored further.

**Key Words**: Cardiac hypertrophy, Late embryogenesis abundant protein, HSP22 protein, Homeostasis



## Introduction

Recently, there has been growing interest in the relationship between cardiac diseases and diet. A long-term, high-salt (NaCl) diet leads to chronic hypernatremia and overall cardiac hypertrophy[1]. Left ventricular hypertrophy increases the possibility of arrhythmia and even sudden cardiac death[2]. In addition to a high-salt diet, angiotensin II (Ang II) is a well-known factor that induces cardiac hypertrophy through the renin-angiotensin system (RAS), and

it has been used to produce models for cardiac hypertrophy[3,4]. However, the direct cellular effects of a high-NaCl diet are not yet clearly understood.

In cardiac hypertrophy, upregulation of early gene expression occurs, such as the expression of sHSP[5]. Moreover, late embryogenesis abundant (LEA) proteins have similar roles in development, and they are classified into groups, according to their sequence and function[6,7]. LEA proteins in group 3 (LEA3), found in *Artemia*, play protective roles during hypersalinity[8]. sHSP and LEA3 have similar protective functions, when an organism faces hyperosmolality and various stresses[6]. However, there is no previous study that shows the relationship between sHSP and LEA proteins.

HSP22 (heat shock protein 22, HspB8, and H11 kinase) is a sHSP that is expressed abundantly in the heart. HSP22 is a mediator of cardiac hypertrophy, and it plays a key role in the regulation of apoptosis, development, and intracellular $Ca^{2+}$ ions[9-14]. The role of HSP22 in cardiac hypertrophy in chronic hypernatremia is currently unknown. Therefore, the aim of the present study was to establish an *in vitro* hypernatremic model with cardiac cellular hypertrophy, to demonstrate the effectiveness of this model by comparing 2 hypertrophic factors (high NaCl and Ang II), and to identify the LEA like-properties of HSP22 in these 2 different cellular models.

## Methods

### 1. Cell Culture

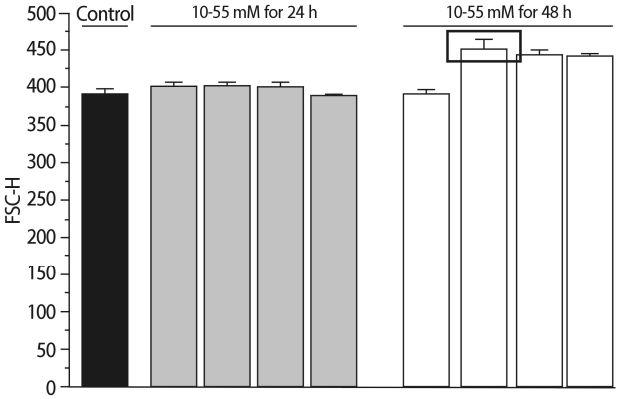
The rat cardiomyoblast-derived H9C2 cell line was purchased from the Korean Cell Line Bank. The H9C2 cells were maintained in Dulbecco's modified Eagle's medium supplemented with fetal bovine serum (FBS; 10%), penicillin (100 $\mu$g/mL), and streptomycin (100 $\mu$g/mL) (DMEM) in 5% $CO_2$ humidified air at 37℃.

### 2. *In vitro* hypernatremic model

The added concentration and maintenance time for NaCl was selected using the forward side scatter pulse height (FSC-H) values from flow cytometry (Table 1, Supplement 1). Based on this, 30 mM NaCl was added to the DMEM. The media were filtered through 0.4 $\mu$m filters. Cells were maintained for 48 h to produce the *in vitro* hypertrophic models.

### 3. Flow cytometric analysis

Suspended H9C2 cells in PBS (phosphate-buffered saline) were used. To exclude osmotic effects, the cells were incubated in normal osmotic PBS for 30 min. After incubation, 10,000 cells were automatically analyzed by FSC-H to measure the cell size and side scatter pulse height (SSC-H) in order to study the intracellular granules (FACS Calibur™).




**Supplement Data 1.** The size of cells changed. Cell sizes were measured and compared with the FSC-H values.

**Table 1.** Conditions for the induction of cellular hypertrophy

| | 10 mM | 30 mM | 40 mM | 55 mM |
|---|---|---|---|---|
| 24 h | 381.42±4.40 | 378.98±4.62 | 368.51±3.17 | 354.07±3.77 |
| 48 h | 383.19±2.75 | **424.48±17.71** | 392.48±25.21 | 390.32±15.16 |
| | | Control: 372.31±15.28 | | |

In a pilot study, FSC-H values changed in high NaCl conditions. The increase in the size of cells was the highest in the 48 h and 30 mM NaCl group.

### 4. Confocal analysis

Cells were fixed in -20℃ methanol for 5 min at room temperature (RT). The cells were washed 3 times with ice-cold PBS and then blocked with 10% FBS in PBS for 30 min at RT. After blocking, the cell nuclei were stained with 1 μg/mL of 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI) in 3% FBS and PBS for 30 min at RT. After staining with DAPI, the images were visualized with a confocal microscope and a coupled image analysis system (Nikon).

### 5. HSP22 sequence homology analysis

A search of the amino acid sequences of a conserved domain of HSP22 and various LEA3 proteins was conducted using BLAST[15]. Utilizing this program, the amino acid sequences in the conserved domains were compared between HSP22 and LEA3[16].

### 6. Cell viability

The trypan blue exclusion test was performed[17]. The results were calculated using the following equation: (*the total number of viable cells/the total number of cells*)×100.

### 7. Real-time reverse transcriptase PCR (RT-PCR) and DNA gel electrophoresis

Total RNA was extracted. The RNA quantity and quality were measured using the 260/280 ratio obtained from a Nano-drop spectrophotometer. Using RT-PCR, cDNA was synthesized from RNA. For the analysis of mRNA expression, forward and reverse primers were used (Table 2). One μL of each set of primers was used with TOPreal™ qPCR premix (10 μL), nuclease-free water (20 μL), and a cDNA sample (1 μL). The Tm value of the primers was 58℃. After an initial 10 min at 95℃, 45 cycles of the following protocol were completed: 95℃ for 10s, 60℃ for 15s, and 72℃ for 30s. The fold changes of HSP22 were compared with those of a house keeping gene (18s ribosomal RNA (rRNA)). The data were analyzed using the $\Delta C_t$ method[18]. To confirm the primer activities, an ethidium bromide-stained agarose gel (2%) was used for DNA gel electrophoresis. The size of the HSP22 product was 129 base pairs (bp), while that of the 18s rRNA product was 135 bp.

### 8. Intracellular $Ca^{2+}$

The cells were loaded with 2 mM of the fluorescence probe Calcium Green-1™ in DMEM for 2 h. Cells were washed twice with PBS, and 10,000 cells were analyzed using flow cytometry. Single cells were observed under a confocal microscope after fixation with ice-cold methanol.

### 9. Statistics

All data were expressed as the mean±S.E., and n indicated the number of cells used. For all tests, cells were obtained from a minimum of 3 different culture dishes for each treatment and experiment. Analysis of the data was conducted using Student's t-test and one-way ANOVA. $p<0.05$ was considered to be significantly different in the statistical analyses.

## Results

### 1. Supplementation with 30 mM NaCl and 1 μM Ang II for 48 h affects the size of cells without inducing abnormal cytological changes

To compare the characteristics of the *in vitro* hypernatremic model, an Ang II-induced hypertrophic model (Ang II model) was used, because treatment with Ang II induces cellular hypertrophy in H9C2 cells, which mimics the hypertrophic responses of primary cardiomyocytes[3]. First, the FSC-H values significantly increased following supplementation with 30 mM NaCl ($p<0.05$) and

Table 2. Primer sequences

| | | |
|---|---|---|
| Ribosomal 18s RNA | Forward: TTG GTG GAG CGA TTT GTC TG | Reverse: CTC AAT CTC GGT GGC TGA AC |
| HSP22 | Forward: TAC CTG CTG AGG GTA GGA AC | Reverse: TCC CTG ACA CTT CCA CGT AT |

1 μM Ang II for 48 h ($p<0.01$) (Figs. 1 and 2), indicating an increase in the size of the cells. The mean of the FSC-H was 391.21±6.63 for the control group. The mean of the FSC-H for the 24 h group with 30 mM NaCl did not change. However, the mean of the FSC-H for 48 h was 416.49±17.08. The mean FSC-H value was 356.77±9.26 for the control group. The mean of the FSC-H for the 24 h group with 1 μM Ang II did not exhibit a statistically significant change. However, the mean of the FSC-H value for the cells maintained for 48 h was 375.09±7.61. There were several factors that could have affected the control samples. For this reason, different controls were used in each experiment to ensure an accurate comparison. The difference in values was more evident for the supplementation with NaCl. Second, SSC-H was constant among the controls after treatment for 24 h and 48 h with each hypertrophy-inducing agent (Fig. 3). Third, the nuclear and cell morphologies were similar among the controls and the 2 models under confocal microscopy with DAPI staining (Supplement 2). Our hypertrophic model, induced by the addition of NaCl, contained less granular content in the cytoplasm than other models. The results indicated that the addition of 30 mM NaCl did not strongly affect the cells. Finally, the cell viability was exam-

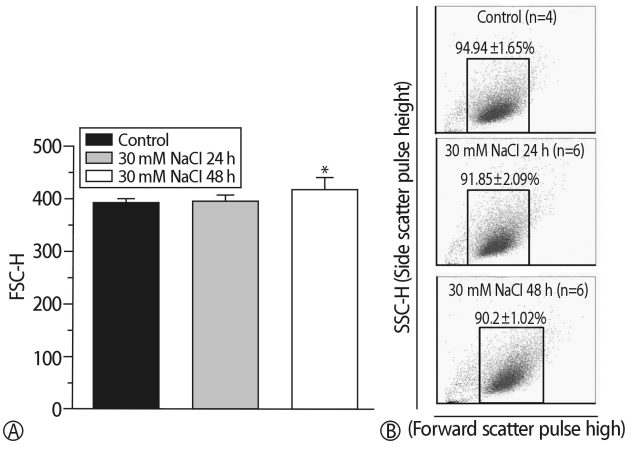



**Fig. 1.** Size of the in vitro hypernatremic model. (**A**) Cell size was increased by supplementation with 30 mM NaCl for 48 h (*$p<0.05$). (**B**) A right shift in the 30 mM NaCl for 48 h group was found.

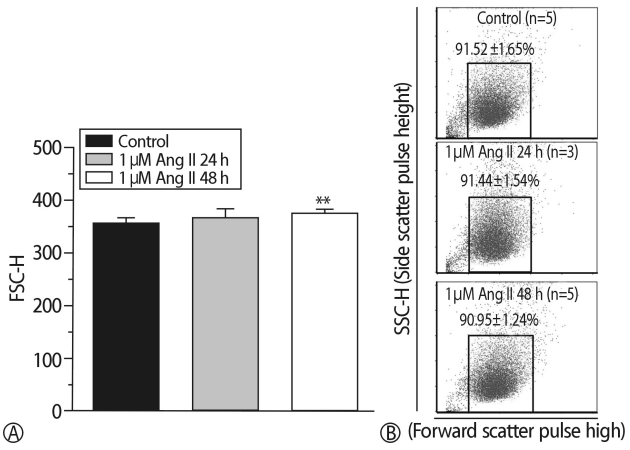



**Fig. 2.** Ang II model was confirmed. (**A**) Hypertrophy was induced by supplementation of 1 μM Ang II for 48 h (**$p<0.01$). (**B**) Right shift in the Ang II model was found.

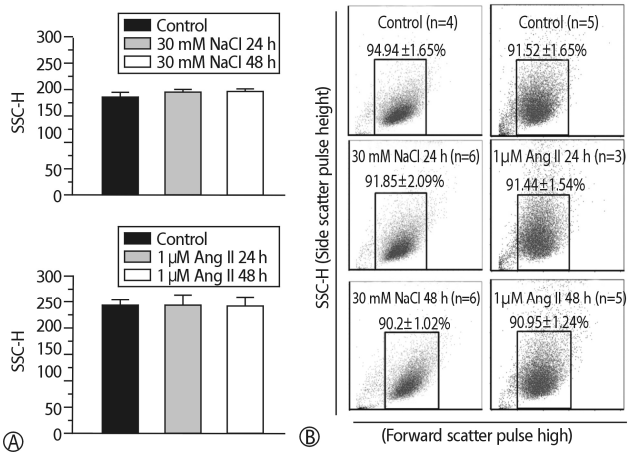



**Fig. 3.** Abnormal granule was confirmed in two different hypertrophic models. (**A**) No changes, (**B**) No shifts in SSC-H were found in two models and each control group.

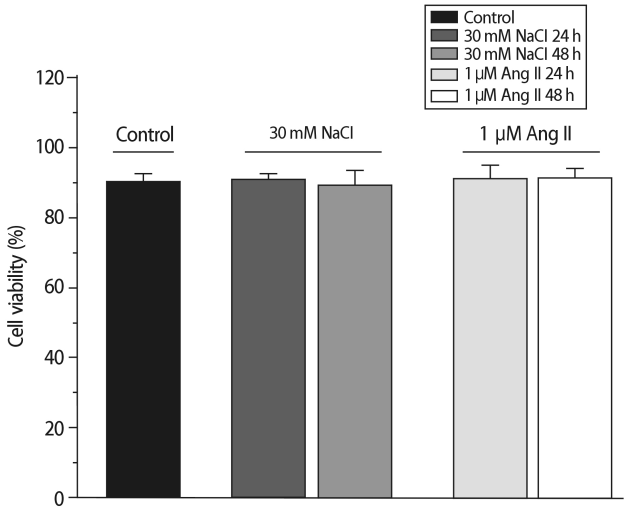
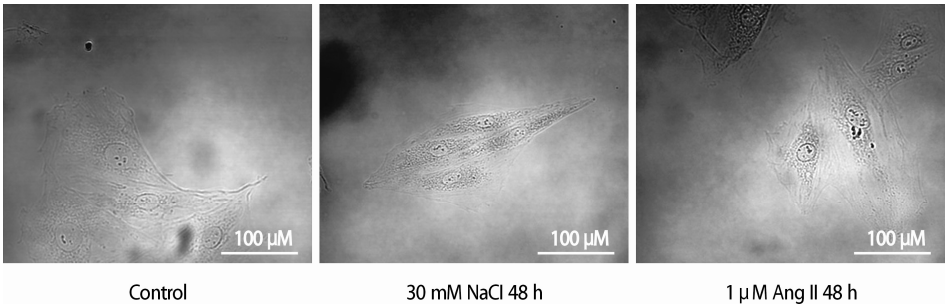



**Fig. 4.** Cytotoxic effects of NaCl and Ang II were compared. Cell viabilities were not statistically different from the value of the control.




**Supplement Data 2.** Morphology of cells. Abnormal cell and nuclear morphologies were not observed among the Control and two hypertrophic models.

ined (Fig. 4). For all of the groups, there were no changes in the cell viability. Therefore, this model did not exhibit abnormal morphological changes, and the cellular hypertrophy induced by these conditions was termed the *in vitro* hypernatremic model.

## 2. Different mRNA expression patterns of HSP22 between the 2 different cellular models

RT-PCR was conducted to compare the mRNA expression patterns after exposure to Ang II or NaCl for 24 or 48 h. HSP22 was compared with a house-keeping gene. The mRNA expression of HSP22 did not increase after treatment with Ang II for 24 h, but the expression of HSP22 increased after 48 h. In contrast to the Ang II model, the expression of HSP22 in the *in vitro* hypernatremic model gradually decreased in a time-dependent manner (Fig. 5). After these experiments, DNA gel electrophoresis was used to confirm the product of each primer set used for RT-PCR (Supplement 3). The results indicated that the design of the primers and PCR analysis were conducted appropriately.

## 3. Amino acid sequence homology between HSP22 and LEA3

A gradual decreasing pattern, in response to high NaCl, has been shown for LEA3 in *Artemia*[19]. Based on the similarity of the decreasing patterns for LEA3 in *Artemia* and HSP22, a comparison of the amino acid sequences was conducted. LEA3 proteins have several types of conserved domains (Table 3)[20]. The sequences of these conserved domains were also compared. The consensus sequence of HSP22 was found at residues 108-128, which

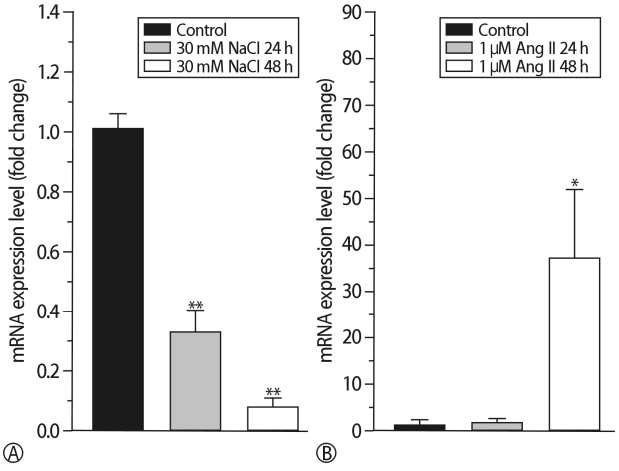
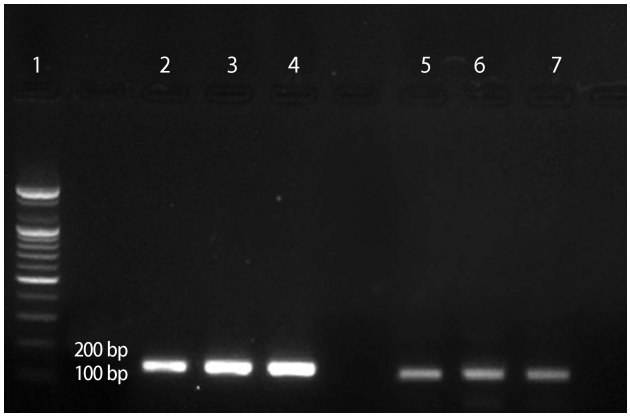



**Fig. 5.** mRNA expression of HSP22 and NPPa in two hypertrophic models. (**A**) In the NaCl group, mRNA expression of HSP22 was significantly decreased in a time-dependent manner. (**B**) HSP22 in the 1 μM Ang II for 48 h group (*p<0.05, **p<0.01, ***p<0.001).




**Supplement Data 3.** To validate the primers, amplified cDNA of the Control group was confirmed by DNA gel electrophoresis. The product sizes were 129bp (HSP22), and 135bp (16s rRNA). Lane.1 is Marker, Lane.2, 3, 4 are 18s rRNA, and Lane. 5, 6, 7 are HSP22.

**Table 3.** Consensus amino acid sequences of the different motifs in LEA3

| Motif | Sequence | Motif | Sequence |
|---|---|---|---|
| 1 | G G V L Q Q T G E Q V<br>S F S A K | 1[a] | T A E K A G E Y K D Y<br>A |
| 2 | A A D A V K H T L G M<br>K E N F<br>T | 4[a] | T V E K A K E A K D T<br>A Q T R T A<br>M |
| 3[a] | T A Q A A K D K T S E<br>S E Q E | 2[a] | A Y E K A G S A K D M<br>D A |
| 5[a] | A T E A A K Q K A S E<br>T A Q T E A | 3[a] | A A Q K A K D Y A G D<br>S E D<br>T S |
| 4 | S Y K A G E T K G R K T<br>R A K A<br>Q | 5 | E S W T E W A K E K I<br>A G D |

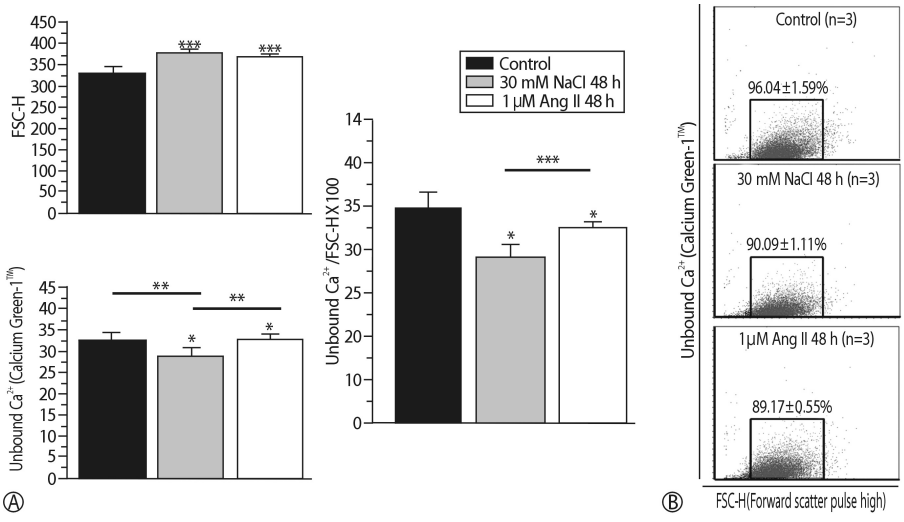



**Fig. 6.** Average intracellular free $Ca^{2+}$ concentration in two hypertrophic models. (**A**) FSC-H increased in the Ang II model and further increased in the *in vitro* hypernatremic model, and fluorescent intensity increased in the Ang II model and decreased in the in vitro hypernatremic model. To sum up, the level of free $Ca^{2+}$ in the region on the cell decreased in the Ang II model and further decreased in the *in vitro* hypernatremic model when fluorescent intensity was corrected to compensate the difference of cell size. (**B**) The two hypertrophic models shifted to the right and down. In *in vitro* hypernatremic model, it was more evident (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

corresponds to residues 117-137 of LEA3 from *Artemia*. The identities were 33% (7/21); 57% (12/21) were positive, there were 0 gaps, and the expectation was 3.9. The similarity was meaningful, because part of the $\alpha$-crystalline domain of HSP22 was similar to 3[a] motif sequence of LEA3. The similar expression patterns and amino acid sequences for HSP22 and LEA3 indicated that HSP22 might have an LEA-like function[20].

## 4. HSP22 affects the intracellular homeostasis of free $Ca^{2+}$

To determine the roles of HSP22 in hypertrophy, the intracellular $Ca^{2+}$ level was compared using a $Ca^{2+}$-binding dye for the control; the FSC-H increased for the 2 hypertrophic models ($p<0.001$). Unbound $Ca^{2+}$ increased in the Ang II model ($p<0.05$). However, compared

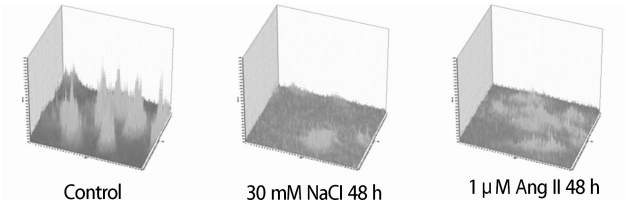



**Fig. 7.** Analysis of the single cell to show the intracellular free $Ca^{2+}$ level. $Ca^{2+}$ concentration decreased in the Ang II model and decreased further in the in vitro hypernatremic model.

with the Ang II model, $Ca^{2+}$ significantly decreased in the *in vitro* hypernatremic model ($p<0.01$). To compensate for the size of the cells using the equation: *unbound* $Ca^{2+}$*/FSC-H×100*, the corrected $Ca^{2+}$ decreased in the Ang II model ($p<0.05$), and the decrease in $Ca^{2+}$ was more evident with NaCl ($p<0.001$) (Fig. 6). The corrected result showed that size of the 2 hypernatremic models increased as the intracellular free $Ca^{2+}$ decreased. Moreover, $Ca^{2+}$ was confirmed in a single cell (Fig. 7). The results indicated that the role of HSP22 in cellular hypertrophy is related to impaired intracellular $Ca^{2+}$ homeostasis.

## Discussion

Hypertrophy in cardiomyocytes is a key factor in the pathogenesis of cardiac diseases, such as heart failure, arrhythmia, and sudden cardiac death[21]. Many previous studies have demonstrated the causes of hypertrophy, including changes in metabolism, RAS, and $Ca^{2+}$-dependent transcription factors[22-27]. However, the mechanism of cellular hypertrophy, induced by a chronic high-NaCl diet, has not been elucidated.

Recently, the re-induction of cardiac embryonic genes, through a process called the "immediate early gene program," has been reported in several studies using cardiac disease models[5,28-30]. sHSPs are cardiac embryonic genes that play protective roles in the heart[31,32]. LEA proteins are also known to be protectors and regulators of cellular development in various organisms[6,20]. In particular, the LEA3 proteins protect the cell during changes in osmolality[8]. Using the BLAST program, similar sequences that protect against osmotic damage were found in both HSP22 and LEA3 from *Artemia*. The $\alpha$-crystalline domain is an important part of the sequence of HSP22[33]. The homology identified in the $\alpha$-crystalline domain was term ed "LEA-like-HSP22." All of these data imply that proteins that regulate development, such as sHSPs and LEA, play key roles in the process of hypertrophy under conditions of high osmolality. Specifically, LEA-like-HSP22 has diverse functions in the heart[9-13,34].

Having been incubated in normal osmotic PBS for 30 min, an increase in FSC-H was sufficient to indicate that hypertrophy had been induced in our *in vitro* hypernatremic model. The lack of abnormal changes in SSC-H, viability, and morphology across the *in vitro* hypernatremic model, control, and Ang II model shows that our model is not negatively affected by the addition of NaCl. In a previous study, an increase in SSC-H was observed in damaged cells[35]. Our hypernatremic conditions could more effectively increase the size of cells than the conditions obtained with Ang II, because the increase in FSC-H in our model was greater than that observed for the Ang II model. Although excitation-contraction coupling (E-C coupling) by action potentials is not found in H9C2 cells, previous studies have shown that the intracellular $Ca^{2+}$ can be changed and that $Ca^{2+}$ can be a mediator of cellular hypertrophy in H9C2 cells[36-42]. Therefore, the intracellular $Ca^{2+}$ homeostasis is important in H9C2 cells.

With our model, our group anticipated that HSP22 would increase to protect the cells in the high-NaCl environment. Contrary to our expectations, HSP22 decreased in our model, and this trend was not observed with the Ang II model. This result suggests that HSP22 may participate in the protective process of cardiac hypertrophy with chronic hypernatremia and the protective process of intracellular $Ca^{2+}$ homeostasis. This agrees with our finding that the $Ca^{2+}$ in the hypertrophic H9C2 cells changed with the different HSP22 expression levels, because $Ca^{2+}$ is used as a second messenger in the heart, and $Ca^{2+}$ has important functions, including the regulation of metabolism, E-C coupling, and hypertrophic processes[43-46]. Our results indicate that HSP22 participates in the intracellular $Ca^{2+}$ homeostasis, because in our model, the impaired, intracellular free $Ca^{2+}$ homeostasis and an increased cell size were more evident with the decreased HSP22 expression than other for the groups.

In summary, the expression of HSP22 was significantly

different in the 2 hypertrophic models, and it is important for the maintenance of the intracellular $Ca^{2+}$ homeostasis in H9C2 cells. However, the present study had several limitations, including the properties of HSP22 in contractible cardiomyocytes and the direct relationship between cardiac hypertrophy, HSP22, and $Ca^{2+}$ in the hypertrophic process. These limitations need to be explored.

## Conclusion

We conclude that a high-NaCl condition induces cardiac cellular hypertrophy, due to impaired intracellular $Ca^{2+}$ homeostasis. It is related to changes in the LEA-like HSP22 expression pattern. This observation can be used for the development of new therapies that can treat cardiac hypertrophy, induced by a chronic high-NaCl diet. However, the protection of the intracellular $Ca^{2+}$ homeostasis with a regulated LEA-like HSP22 expression pattern must be confirmed *in vivo* and in patients.

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