

# Effects of a Proteasome Inhibitor on Cardiomyocytes in a Pressure-Overload Hypertrophy Rat Model: An Animal Study

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**Background:** The ubiquitin-proteasome system (UPS) is an important pathway of proteolysis in pathologic hypertrophic cardiomyocytes. We hypothesize that MG132, a proteasome inhibitor, might prevent hypertrophic cardiomyopathy (CMP) by blocking the UPS. Nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) and androgen receptor (AR) have been reported to be mediators of CMP and heart failure. This study drew upon pathophysiologic studies and the analysis of NF- $\kappa$ B and AR to assess the cardioprotective effects of MG132 in a left ventricular hypertrophy (LVH) rat model. **Methods:** We constructed a transverse aortic constriction (TAC)-induced LVH rat model with 3 groups: sham (TAC-sham, n=10), control (TAC-cont, n=10), and MG132 administration (TAC-MG132, n=10). MG-132 (0.1 mg/kg) was injected for 4 weeks in the TAC-MG132 group. Pathophysiologic evaluations were performed and the expression of AR and NF- $\kappa$ B was measured in the left ventricle. **Results:** Fibrosis was prevalent in the pathologic examination of the TAC-cont model, and it was reduced in the TAC-MG132 group, although not significantly. Less expression of AR, but not NF- $\kappa$ B, was found in the TAC-MG132 group than in the TAC-cont group ( $p < 0.05$ ). **Conclusion:** MG-132 was found to suppress AR in the TAC-CMP model by blocking the UPS, which reduced fibrosis. However, NF- $\kappa$ B expression levels were not related to UPS function.

**Key words:** 1. Cardiomyopathy, hypertrophic  
2. Ubiquitins  
3. Proteasome inhibitors  
4. MG132  
5. Receptors, androgen  
6. NF-kappa B

## Introduction

Protein synthesis and proteolysis can be increased by abnormal ventricular hypertrophy. The ubiquitin-proteasome system (UPS) is the principal mechanism for protein degradation in the cytoplasm and the nucleus. Three enzymes that are required for the conjugation of ubiquitin to target proteins have been

isolated. The ubiquitinated proteins are then transferred to the proteasome and degraded [1,2]. It has been suggested that the inhibition of proteasomes is associated with cardiovascular disease progression [3,4]. Androgen receptors (ARs) have been reported to be a mediator of cardiac hypertrophy [5], and require proteasome activity for transcriptional activity in prostate cancer cells [6]. In contrast, nuclear fac-

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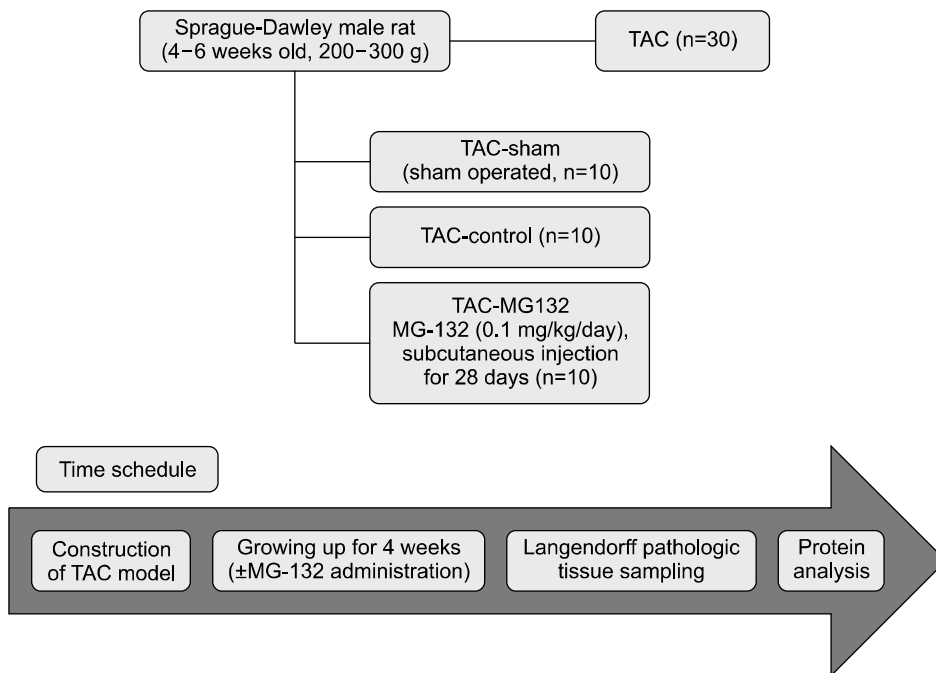


Fig. 1. Schematic diagram of the experimental design. TAC, transverse aortic constriction.

tor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) is a protein complex that controls DNA transcription and cytokine production [7]. NF- $\kappa$ B is involved in cellular responses to many kinds of stimuli, and it plays an important role in regulating cellular responses [8]. Furthermore, NF- $\kappa$ B has been reported to be significantly activated in heart failure [9].

In this study, we assessed the cardioprotective effects of the proteasome inhibitor MG132 in a pressure-overload ventricular hypertrophy rat model. Hemodynamic studies, observations of pathologic differences, and protein analyses of NF- $\kappa$ B and AR were performed to characterize the effects of MG132.

## Methods

### 1) Animals

In total, 30 Sprague-Dawley male rats (4-6 weeks old, 200-300 g) were housed at 20°C±2°C and 55%±20% humidity with 12-hour light/dark cycles and free access to food and water in the Animal Care Facility at Korea University Ansan Hospital, Korea. This study was approved by the Committee of Animal Care of Korea University (IRB no. KUIACUC-2015-7) and conformed to the Guide for the Care and Use of Laboratory Animals by the US National

Institutes of Health (NIH publication no. 85-23, revised 1996).

### 2) Experimental design

Left ventricular hypertrophy (LVH) was induced using transverse ascending aortic constriction (TAC). The animals were divided into 3 groups: sham (TAC-sham, n=10), control (TAC-cont, n=10), and MG132 administration (TAC-MG132, n=10). In the TAC-sham group, only skin incision and closure, without aortic constriction, were performed. The control group (TAC-cont) consisted of the hypertrophy model of TAC without MG132 administration. The TAC-MG132 group received MG132 subcutaneously (0.1 mg/kg/day) for 4 weeks after the TAC model construction. A schematic diagram of the experimental design is shown in Fig. 1.

### 3) Construction of the LVH (TAC) rat model

The TAC rat model was constructed using transverse ligation around the ascending aorta. After sedation with 5% enflurane-O<sub>2</sub> (1 L/min) in a canister, an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg) was performed [10]. Intubation and mechanical ventilation (Harvard ventilator 683; Harvard Apparatus, Holliston, MA, USA) were applied according to the method described by Rivard

et al. [11]. After antiseptic preparation, a left second intercostal incision was made. After careful dissection and lifting of the thymus, the ascending aorta was identified. A curved 16-gauge angiocatheter was tied around the ascending aorta, and was then angiocatheter was removed. The outer diameter of the constricted ascending aorta was approximately 16-gauge (1.29 mm). The mean external diameter of the ascending aorta in normal rats has been reported to be 2.16 mm by Feng et al. [12]. Therefore, the aortic diameter in the TAC model was approximately 60% of the native ascending aortic diameter. MG-132 was injected for 28 days, starting on the first postoperative day, in the TAC-MG132 group. For the other groups, subsequent experiments were performed 4 weeks after TAC or TAC-sham construction.

#### **4) Hemodynamic study using the Langendorff procedure**

After sedation as mentioned above, the heart was excised and connected to a Langendorff apparatus (size 3, type 830; Hugo Sachs Elektronik, March-Hugstetten, Germany) as in our previous studies [13]. A water-filled balloon catheter was inserted into the left ventricle via the left atrial appendage and connected to a pressure transducer (DX-360; Nihon Kohden, Tokyo, Japan). After 15–20 minutes of calibration, hemodynamic data (pressure change per second [dP/dT], the heart rate, and peak left ventricular pressure [peak LVP]) were recorded using PowerLab/4SP software (AD Instruments, Mountain View, CA, USA).

#### **5) Pathologic study**

After the hemodynamic study, the hearts were weighed, cross-sectioned, and fixed with formaldehyde for microscopic examination. Cardiac tissue has an extracellular collagen network to provide cardiac contractility and strength. In most cardiac diseases, increased collagen deposition, or fibrosis, is observed [14]. Masson trichrome (MT) staining was performed to distinguish collagen fibers from cardiac muscular tissue.

#### **6) Protein analysis**

**(1) Real-time polymerase chain reaction:** After the Langendorff experiments, tissues from the left ventricle were collected and quick-frozen in liquid nitro-

gen. To identify the gene expression of AR and NF- $\kappa$ B, quantitative real-time polymerase chain reaction (PCR) was performed. Total RNA was extracted from 100 mg of left ventricular tissue using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified using a NanoDrop spectrophotometer. For the complementary DNA (cDNA) synthesis, 2 ng of RNA was used in the Reverse Transcription System (Promega, Madison, WI, USA). According to the manufacturer's protocol, the quantitative PCR on the synthesized cDNA was performed using Light Cycler Fast Start DNA Master SYBR Green I (Roche, Indianapolis, IN, USA) in the Light Cycler 1.5 system (Roche). The AR gene was amplified using the primers 5'-AAA GGT CTT TCC CTG GAC GA-3' (sense) and 5'-TCT CAC CTT CCA ACC CTT TG-3' (antisense). The NF- $\kappa$ B gene was amplified using the primers 5'-GGA GAT GGC CCA CTG CTA TC-3' (sense) and 5'-TTT ACA GTG TGG GGA ACC GC-3' (antisense). The quantitative gene expression of AR and NF- $\kappa$ B was defined by their ratio to the expression of  $\beta$ -actin. The  $\beta$ -actin gene was amplified using the primers 5'-GGT CCT AGC ACC AAT GAA GA-3' (sense) and 5'-ATC TGC TGG AAG GTG GAC AG-3' (antisense).

**(2) Protein expression of androgen receptor using western blotting:** Frozen left ventricular tissue was homogenized in a radioimmunoprecipitation assay buffer, and following centrifugation (1,200 rpm for 10 minutes), the supernatant was adjusted to a protein concentration of 2  $\mu$ g/ $\mu$ L using the Bradford assay (Bradford reagent; Sigma, St. Louis, MO, USA). Sodium dodecyl sulfate (SDS) electrophoresis was performed in an 8% SDS acrylamide gel; then, proteins were transferred onto polyvinylidene difluoride membranes. For blocking, 5% skim milk was used for 1 hour. AR was detected using a primary AR antibody (1:1,000 dilution) followed by the secondary anti-AR antibody (1:5,000 dilution). For the control, glyceraldehyde 3-phosphate dehydrogenase primary antibody (1:4,000 dilution) and secondary antibody (1:5,000 dilution) were used. After reacting with an enhanced chemiluminescence solution, image analysis was performed using the ImageJ program (National Institutes of Health, Bethesda, MD, USA).

**(3) Enzyme-linked immunosorbent assay of NF- $\kappa$ B activity:** NF- $\kappa$ B is a complex formed by the Rel-like domain, including RELA/p65, RELB, NFKB1/p105, NFKB1/p50, REL, and NFKB2/p52, of which p65 and

Table 1. The heart and body weight and the results of hemodynamic study in each subgroup

Variable	Group	Value	One-way (F/p-value)	Multiple comparison	p-value
HW (g)	TAC-sham	1.33±0.22	21.928/0.000	TAC-sham < TAC-cont	< 0.001
	TAC-cont	2.13±0.38		TAC-sham < TAC-MG132 (Tamhane T2)	
	TAC-MG132	1.99±0.24			
BW (g)	TAC-sham	341.0±95.0	16.466/0.000	TAC-sham < TAC-cont	0.001
	TAC-cont	498.5±40.6		TAC-cont > TAC-MG132 (Tamhane T2)	
	TAC-MG132	415.1±25.1			
HW/BW (%)	TAC-sham	0.41±0.70	3.464/0.046	-	-
	TAC-cont	0.43±0.07			
	TAC-MG132	0.48±0.05			
Heart rate (bpm)	TAC-sham	184.2±45.31	1.554/0.234	-	-
	TAC-cont	154.1±24.3			
	TAC-MG132	178.3±36.2			
Peak left ventricle pressure (mm Hg)	TAC-sham	115.4±36.7	3.169/0.062	-	-
	TAC-cont	126.6±40.3			
	TAC-MG132	165.6±50.4			
dP/dT (mm Hg/sec)	TAC-sham	1,777.2±708.1	4.463/0.024	TAC-cont < TAC-MG132 (Scheffe)	0.042
	TAC-cont	1,631.5±719.5			
	TAC-MG132	2,875.6±1,244.9			

Values are presented as mean±standard deviation. Between-group comparisons were performed with 1-way analysis of variance and multiple comparisons.

HW, heart weight; BW, body weight; dP/dT, pressure change per second; TAC, transverse aortic constriction; cont, control.

p50 are the most abundant [15]. NF- $\kappa$ B controls DNA transcription by binding to DNA. For the identification of NF- $\kappa$ B p65 activity, 40 mg of left ventricular tissue was extracted using the NE-PER nuclear and cytoplasmic extraction reagent (Thermo Fisher Scientific, Rockford, IL, USA) according to the manufacturer's protocol. The NF- $\kappa$ B p50/p60 transcription factor assay kit (Abcam, Cambridge, UK) was used according to the manufacturer's protocol to detect the DNA binding activity of NF- $\kappa$ B.

### 7) Statistical analysis

All data are presented as mean±standard deviation. Comparisons between the 2 groups were carried out using the Mann-Whitney test. Comparisons among 3 groups were performed with 1-way analysis of variance and multiple comparisons. Differences were considered significant at  $p < 0.05$ .

## Results

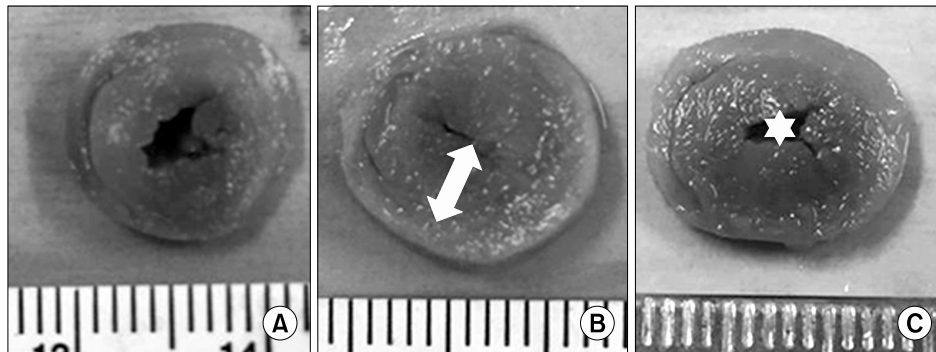
### 1) Hemodynamic study

Differences in dP/dT were found among the study

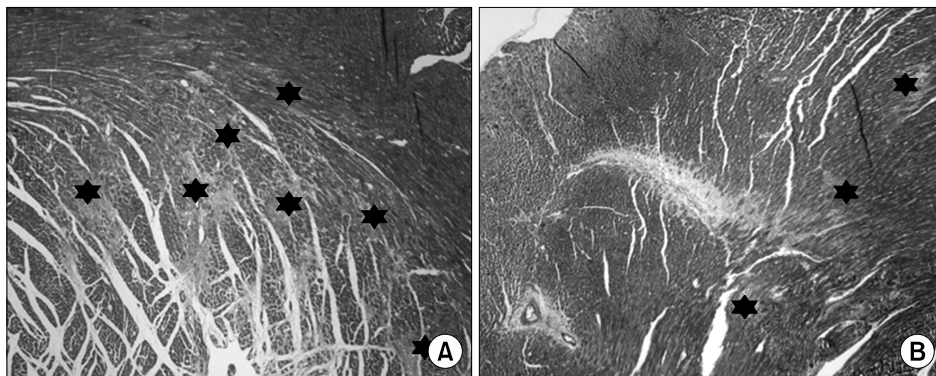
groups. The dP/dT of the control group was lower than that of the sham group. In the MG132 subgroup, dP/dT was greater than in the control subgroup. In our study, we used the pressure change per second to evaluate left ventricular function as a relative change value, because the calibration of the standard left ventricular filling pressure has been found to have some limitations in dozens of rat experiments, leading to potential bias in the data. These findings were not statistically significant, as shown in Table 1. Additionally, the heart rate and peak LVP did not significantly vary among the groups.

### 2) Pathologic results

The heart weight was greater in the TAC-cont group than in the TAC-sham group (2.13±0.38 g in the TAC-cont group and 1.33±0.22 g in the TAC-sham group;  $p < 0.001$ ). However, the TAC-MG132 group did not show a significantly different heart weight compared to the TAC-cont group, although the mean value was lower in the TAC-MG132 group (2.13±0.38 g in the TAC-cont group and 1.99±0.24 g in the TAC-MG132 group;  $p = 0.427$ ) (Table 1).



**Fig. 2.** Gross appearance of the left ventricular cross sections in each group. The TAC-cont group displayed definitively hypertrophied left ventricles with smaller ventricular cavities than the TAC-sham group. The TAC-MG132 group showed less ventricular thickness than the TAC-cont group. (A) TAC-sham. (B) TAC-cont. The cross-section of the heart showed a nearly obstructed left ventricular cavity with a hypertrophied left ventricular wall (white arrow). (C) TAC-MG132. The left ventricular wall thickness decreased after MG132 administration. The ventricular cavity (white asterisk) was larger than in the TAC-cont group and smaller than in the TAC-sham group. TAC, transverse aortic constriction; cont, control.



**Fig. 3.** Fibrotic lesions. The ventricular tissue appears blue with Masson trichrome staining because of collagen-rich fibrotic lesions (black asterisk). The TAC-MG132 group showed less fibrosis than the TAC-cont group. (A) TAC-cont ( $\times 40$ ). Multiple fibrotic lesions were found in the TAC-cont group. (B) TAC-MG132 ( $\times 40$ ). In the TAC-MG132 group, fewer fibrotic lesions (black asterisk) were found than in the TAC-cont group. TAC, transverse aortic constriction; cont, control.

The ratio of heart to body weight was not significantly different between the groups. The pressure-overload hypertrophic model was apparent in the gross appearance of heart cross-sections. The TAC-cont group displayed definitively hypertrophied left ventricles with smaller ventricular cavities. In terms of gross appearance, the TAC-MG132 group showed less ventricular thickness than the TAC-cont group (Fig. 2).

MT staining was performed to identify the progression of fibrosis in the left ventricles. The fibrotic lesions were counted (Fig. 3). Although the TAC-cont group showed more fibrotic lesions than the TAC-sham group, this trend did not reach statistical significance. The TAC-MG132 group showed less fibrosis

than the TAC-cont group, but this trend was likewise not statistically significant (Table 2).

### 3) Protein analysis

We compared the gene and protein expression of AR and NF- $\kappa$ B between the control and MG132 groups. Both the gene and protein expression levels of AR were significantly lower in the MG132 group ( $p < 0.05$ ) (Table 3). However, NF- $\kappa$ B showed no significant differences between the control and MG132 groups (Fig. 4). In summary, MG132 administration was effective in decreasing AR expression in pressure-overload hypertrophic cardiomyopathy. However, it had no effect on NF- $\kappa$ B expression.

Table 2. Number of fibrotic lesions in each group

Variable	Group	Value	One-way (F/p-value)	Multiple comparison	p-value
Fibrosis	TAC-sham	1.2±1.2	1.508/0.239	-	-
	TAC-cont	7.4±11.6			
	TAC-MG132	4.5±6.7			

Values are presented as mean±standard deviation. Between-group comparisons were performed with 1-way analysis of variance and multiple comparisons. Although fibrotic lesions were prominent in the TAC-cont and TAC-MG132 groups, no statistically significant difference was observed between the TAC-cont and TAC-MG132 groups.

TAC, transverse aortic constriction; cont, control.

Table 3. The mean levels of gene and protein expression of AR and NF- $\kappa$ B

Variable	TAC-cont	TAC-MG132	p-value
AR, gene	0.006±0.008	0.001±0.001	0.004
AR, protein	0.531±0.172	0.308±0.154	0.013
NF- $\kappa$ B, gene	0.019±0.013	0.067±0.679	0.041
NF- $\kappa$ B, activity	0.239±0.014	0.247±0.0246	0.481

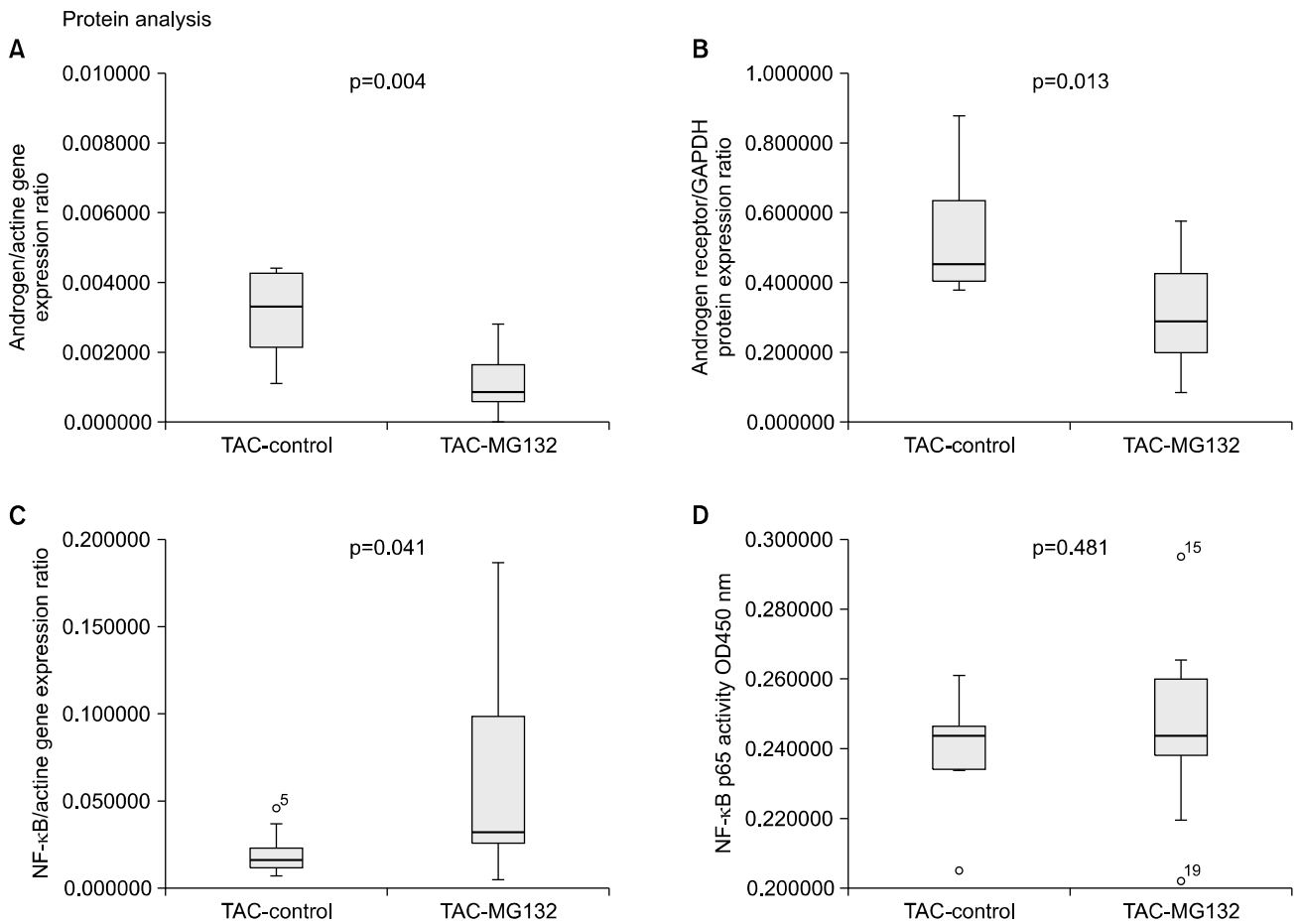
Values are presented as mean±standard deviation. Between-group comparisons were performed with the Mann-Whitney test. AR expression was suppressed in the TAC model by the administration of MG132. However, NF- $\kappa$ B activity was not associated with the administration of MG132.

AR, androgen receptor; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; TAC, transverse aortic constriction; cont, control.

## Discussion

Since its discovery, the UPS has been found to be involved in a wide variety of cellular processes, including antigen processing, apoptosis, the cell cycle and division, and response to stress and extracellular modulators [16]. Approximately 80% of intracellular proteins, including structural and regulatory proteins, are degraded by the UPS [17]. The UPS is a complex structure involving ubiquitin; the E1, E2, and E3 enzymes; and the proteasome. Ubiquitin-activating enzyme (E1) activates free ubiquitin to transfer it to the ubiquitin-conjugating enzyme (E2). Ubiquitin-protein ligases (E3) interact with E2 and substrate proteins, regulating the transfer of ubiquitin to the target protein. Therefore, the E3 ubiquitin ligases provide the specificity of the UPS because the E3 ligase recognizes and targets proteins for degradation [18]. The ubiquitinated proteins are recognized and degraded by the 26S proteasome. Few studies have been published investigating the UPS in the context of cardiac disease models. However, the therapeutic effects of proteasome inhibitors or E3 ligase in-

hibitors have received attention from many investigators [19]. MG132, a type of proteasome inhibitor, is known to suppress cardiomyocyte hypertrophy and to attenuate cholesterol-induced cardiac hypertrophy [20]. Recently, Chen et al. [21] reported that MG132 attenuated pressure-overload hypertrophic cardiomyopathy by modulating protein kinase signals. The E3 ubiquitin ligases, including atrogin-1 and MuRF1, have been found to show increased expression in pathologic cardiac hypertrophy. Inhibition of the 26S proteasome resulted in reduced infarct size and improvement of left ventricular function in an experimental cardiac ischemic-reperfusion injury model [22,23]. This cardioprotective effect was related to the induction of heat-shock proteins or the suppression of NF- $\kappa$ B activity. The suppression of NF- $\kappa$ B has been found to prevent the induction of apoptosis via the inhibition of NF- $\kappa$ B-induced inflammatory intermediates [24,25]. NF- $\kappa$ B is involved in the transcription of DNA, cytokine production, cell survival, and cellular responses to stimuli. Furthermore, NF- $\kappa$ B silencing has been found to prevent cardiac hypertrophy and heart failure [26]. MG132 treatment also decreased NF- $\kappa$ B activity in a diabetic cardiomyopathy mouse model [27]. For these reasons, NF- $\kappa$ B was selected for analysis in this study to assess the effects of MG132 on the inflammatory reaction and apoptosis in pressure-overload ventricular hypertrophy. However, in this pressure-overload hypertrophic model, NF- $\kappa$ B activity did not change after MG132 treatment. NF- $\kappa$ B activation is initiated by degradation of the protein I $\kappa$ B, which occurs via activation of I $\kappa$ B kinase (IKK). The activation of IKK induces the degradation of I $\kappa$ B by ubiquitination and the proteasome. Following the degradation of I $\kappa$ B, NF- $\kappa$ B enters the nucleus and triggers the expression of specific genes [28], including its inhibitor, I $\kappa$ B. This new I $\kappa$ B inhibits NF- $\kappa$ B,



**Fig. 4.** Expression of AR and NF- $\kappa$ B in the TAC groups. We compared the gene and protein expression of AR and NF- $\kappa$ B between the control and MG132 sub-groups. Both gene and protein expression levels of AR were significantly lower in the MG132 sub-group ( $p < 0.05$ ). However, NF- $\kappa$ B did not show a significant difference between the control and MG132 sub-groups. (A) Gene expression of AR between the TAC-cont and TAC-MG132 groups. The gene expression of AR was reduced by MG132 treatment ( $p=0.004$ ). (B) Protein expression of AR between the TAC-cont and TAC-MG132 groups. The protein expression of AR was reduced by MG132 treatment ( $p=0.014$ ). (C) Gene expression of NF- $\kappa$ B between the TAC-cont and TAC-MG132 groups. Gene expression was increased by MG132 treatment ( $p=0.041$ ). (D) NF- $\kappa$ B activity between the TAC-cont and TAC-MG132 groups. NF- $\kappa$ B activity did not change after MG132 treatment ( $p=0.481$ ). AR, androgen receptor; NF- $\kappa$ B, nuclear factor kappa-light-chain-enhancer of activated B cells; TAC, transverse aortic constriction; cont, control.

resulting in an oscillating level of NF- $\kappa$ B [29]. In light of the involvement of NF- $\kappa$ B in cardiac disease and its role in regulating of the UPS, we evaluated the activity of NF- $\kappa$ B in the TAC-LVH model. However, no specificity for NF- $\kappa$ B activity was observed. This may be explained by several possible factors. One is the timing of the Langendorff experiment. The left ventricular tissue was obtained after the Langendorff experiment, and this timing may have influenced NF- $\kappa$ B activity. The second possibility is the oscillating levels of NF- $\kappa$ B, as described above. The third possibility is that NF- $\kappa$ B is not involved in apoptosis or

the inflammatory reaction in this LVH rat model. For reference, Ma et al. [30] reported NF- $\kappa$ B reduction after MG132 injection in an abdominal aortic banding rat model. In our experiment, gene expression increased, but protein activity did not change after 4 weeks of MG132 administration. Therefore, further research is needed to clarify the effect of MG132 on NF- $\kappa$ B.

AR is a nuclear receptor that is activated by androgen hormones. Upon activation, AR is translocated into the nucleus, where it regulates gene expression as a DNA-binding transcription factor [31]. AR medi-

ates cardiac hypertrophy by the receptor-specific hypertrophic responses of testosterone and dihydrotestosterone [5]. Also, it has been reported that proteasome activity involved the transcriptional activity of AR in prostate cancer cells [6]. For these reasons, an analysis of AR was performed, showing that MG132 suppressed AR in the TAC-LVH model. Therefore, the cardioprotective effect of MG132 may be related to the suppression of AR. Alterations in collagen expression in a pressure-overload hypertrophic model due to proteasome inhibition have been reported [32]. In that report, 16 weeks of MG132 administration affected angiotensin II-induced collagen synthesis. In our experiment, 4 weeks of MG132 administration had a similar result, reducing left ventricular fibrosis, although this did not show statistical significance.

We consider that the gross appearance of LVH (confirmed with pathologic specimens of hypertrophied myocardium) and the hemodynamic data can be considered sufficient to confirm the completeness of this animal model. Although this experiment has some limitations regarding the hemodynamic study, the values of dP/dT and peak LVP can be accepted as parameters for assessing the animal model, as discussed in our previous articles [13,33]. Because the pressure change per second is affected by variations in pressure and heart rate, it follows that large alterations of the ventricular pressure and a more rapid heart rate mean greater improvements in ventricular function. Therefore, the high dP/dT observed in the TAC-MG132 group was considered to be reasonable.

In conclusion, MG132 was found to suppress AR via blockage of the UPS in a pressure-overload ventricular hypertrophy rat model. However, NF- $\kappa$ B did not appear to be related to the UPS in this hypertrophic rat model. Further research is needed to clarify the effect of MG132 on NF- $\kappa$ B. Based on our results, we hypothesize that MG132 prevents hypertrophy of the left ventricle by blocking the UPS pathway that induces fibrosis, and that this effect is related to AR.

### Conflict of interest

No potential conflicts of interest relevant to this article are reported.

### Acknowledgments

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