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Basic Science

Upregulation of Myocardial Neutrophil Gelatinase-Associated Lipocalin during Development of Heart Failure Caused by Volume-Overload and the Effect in Regulating Activity of Matrix Metalloproteinase-9

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Background: In patients with heart failure (HF), circulating neutrophil gelatinase-associated lipocalin (NGAL) level is increased, which is considered to be a predictor of mortality or renal outcomes. The expression of NGAL in the heart and kidney and its role in HF remain unclear.

Methods: Aortocaval fistula was created in rats as a model of volume overload (VO)-induced HF.

Results: During the development of HF, NGAL expression was upregulated in the heart but not in the kidney at both transcriptional and translational levels in the compensatory and HF phases, with a similar level in both phases. Cardiomyocytes were identified as the cell type responsible for NGAL expression. Consistent with the myocardial NGAL expression pattern, the plasma NGAL level was increased in both phases, and the level was not significantly different between both phases. We demonstrated the presence of a matrix metalloproteinase (MMP)-9/NGAL complex in cultured medium of cardiomyocytes isolated from volume-overloaded hearts by gelatin zymography. Formation of MMP-9/NGAL complex was shown to enhance the enzymatic activity of MMP-9. We found that early growth response (Egr)-1 was upregulated in the heart in both compensatory and HF phases. In neonatal cardiomyocytes, Egr-1 overexpression induced the gene expression of NGAL, which was dose-dependently suppressed by an interleukin-1 receptor antagonist.

Conclusions: During the development of HF due to VO, NGAL was upregulated in the heart but not in the kidney in both compensatory and HF phases, with a similar expression level. Myocardial NGAL upregulation enhanced MMP-9 activity through formation of the MMP-9/NGAL complex. The expression of myocardial NGAL was regulated by Egr-1.

Key Words: Heart failure • Matrix metalloproteinase-9 • Neutrophil gelatinase-associated lipocalin • Volume-overload

INTRODUCTION

Neutrophil gelatinase-associated lipocalin (NGAL) is a pleiotropic molecule involved in a variety of physiological and pathophysiological processes including metabolic homeostasis, apoptosis, infection, and inflammation.[1] It has been shown to be upregulated in damaged renal tubules and has been identified as a biomarker for

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renal tubular damage.[2-6] Recently, circulating NGAL level has also been demonstrated to be elevated in patients with heart failure (HF). The potential role of circulating NGAL level as a predictor of clinical outcomes in terms of cardiac mortality or worsening of renal function has been tested in numerous studies, however the results have been inconsistent.[7-14] In patients admitted due to acute HF, plasma NGAL has been demonstrated to be a strong prognostic indicator of 30-day outcomes.[8] In a small cohort of 46 elderly patients with HF of varying degrees, baseline NGAL was shown to be a predictor of survival at 2 years.[9] In addition, serum NGAL level was found to be associated with adverse outcome in elderly patients with chronic ischemic HF although this association was lost after multivariate analysis.[10]

The heart and kidney are potential sources of circulating NGAL in HF patients. However, the expression of NGAL in both organs during the development of HF remains unclear. Increased myocardial NGAL expression has been reported in rats with post-myocardial infarction HF.[11] In contrast, myocardial NGAL expression was shown to vary in a porcine model of tachycardia-induced HF, and it was not significantly different from that of the sham control.[15] In patients with HF, information regarding myocardial NGAL expression is scarce. Increased NGAL expression has only been shown in two myocardial samples from HF patients using immunohistochemistry.[11] Moreover, few studies have investigated the renal NGAL expression in patients with HF. It is important to understand the myocardial and renal expressions of NGAL during the development of HF and their correlation with circulating NGAL level for the precise application of circulating NGAL as a marker of cardiac or renal outcomes.

In addition to playing a potential role as an outcome predictor, NGAL may have a further role in the pathogenesis of HF. One potential mechanism by which NGAL may be involved in the progression of HF may be through modification of the extracellular matrix by enhancing the enzymatic activity of matrix metalloproteinase (MMP)-9 through the formation of an NGAL/MMP-9 complex.[7] However whether this complex is present in failing hearts remains unclear.

In this study, we investigated NGAL expressions in the heart and kidney and the changes in circulating NGAL level during the development of volume overload (VO)-induced HF and after correction of VO. We also examined the presence of the NGAL/MMP-9 complex in volume-overloaded hearts and its effect on MMP-9 activity. We further tested the role of early growth response (Egr)-1, a master regulator of inflammatory gene expression,[16] in regulating myocardial NGAL expression.

MATERIALS AND METHODS

Rat model of aortocaval fistula

The aortocaval fistula (ACF) model was created in adult male Sprague-Dawley rats (300 to 350 g). After anesthetization with ketamine (0.2 ml/100 mg i.p.), the abdominal cavity was opened. The inferior vena cava (IVC) and aorta were exposed and clamped right below the renal artery proximally, and right above the iliac bifurcation distally. An 18-gauge needle was used to puncture the lateral wall of the abdominal aorta, right superior to the distal clamped site. The needle was advanced to cross the opposite aortic wall toward the IVC and then penetrate the neighboring wall of the IVC cautiously to avoid puncturing the opposite wall. Finally, the needle was withdrawn gently, and the entry point was sealed with cyanoacrylate glue (Vetbond 3M). Patency of the fistula was confirmed by pulsation and color change in the IVC resulting from the shunt of oxygenated blood from the aorta. A sham operation was done by simply puncturing the lateral wall of the aorta. To close the ACF, the abdomen was incised and the IVC and aorta was exposed. After careful dissection of soft tissue surrounding the vessels, the ACF was identified and clamped using a hemoclip. This study was carried out in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All study protocols of the animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of Chang Gung University (Permit Number: 2009112701).

Echocardiography and hemodynamic studies

Echocardiographic and hemodynamic studies were performed before the animals were sacrificed. M-mode and two-dimensional echocardiography were performed using a 12-MHz probe with a Sonos 5500 echograph (Philips Medical Imaging, Andover, MA). For the hemodynamic study, a microtip pressure-volume catheter

(1.9-F, Model FT212B, Scisense Inc., Ontario, Canada) was used. Signals were connected to a Ponemah ACQ16 acquisition system (DSI Ponemah, Valley View, OH) and analyzed using cardiac pressure-volume analysis software (P3 Plus4.80-SP4; DSI Ponemah).

Purification of cells from the heart

Cardiomyocytes and non-cardiomyocytes were isolated from the rats 2 weeks after the creation of the ACF or sham operation by collagenase perfusion treatment using a Langendorff apparatus. Cells were sedimented and separated by differential centrifugation. The isolated cells were then separated serially through a 38-μm stainless steel sieve twice to yield cardiomyocytes, and then through a 20-μm sieve twice to yield non-cardiomyocytes. Endothelial cells were isolated from the non-cardiomyocyte cell fraction using rabbit anti-mouse CD31 antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and subsequently extracted using a secondary anti-rabbit mouse antibody coupled to paramagnetic microbeads (Thermo Scientific, Fremont, CA). In the cardiomyocyte fraction, > 95% of the cells were monoclonal anti-sarcomeric actin-positive (monoclonal anti-rabbit sarcomeric actin, Sigma-Aldrich, St Louis, MO), > 92% of the cells in the endothelial cell fraction were CD31-positive as assayed by immunocytochemical analysis, and 95% of the cells in the remaining non-cardiomyocyte fraction were vimentin-positive (monoclonal anti-pig vimentin antibody, Thermo Scientific, Fremont, CA).

Isolation and culture of neonatal cardiomyocytes

Primary neonatal cardiomyocytes were isolated from postnatal day 1-2 rats using a Worthington neonatal cardiomyocyte isolation system (Worthington Biochemical Corp. Lakewood, NJ) following the manufacturer's instructions. Cardiomyocytes were cultured in complete Dulbecco's Modified Eagle's Medium (DMEM)-high glucose, with 1% L-glutamine, 1% sodium pyruvate, 10% FBS and 1% penicillin-streptomycin. The experiments were performed 6-8 days after plating. For the transient transfection of Egr-1, cultured neonatal cardiomyocytes at 60-70% confluence were transfected with pCMV6-XL5-EGR-1 (OriGene Technologies. Rockvilles, MD) or with an empty control vector using liposome technology (FuGENE 6; Roche, Indianapolis, IN) according to the manufacturer's protocol. To inhibit the IL-1 pathway, a rat recombinant IL-1 receptor antagonist (R&D Systems, Inc. Minneapolis, MN) was used.

Real-time quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted using a Qiagen RNeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA). The quality and quantity of the total RNA were analyzed using a Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA). Real-time quantitative reverse transcription-polymerase chain reactions (RT-qPCRs) were performed as described previously.[17] The primer sequences used for PCR were as follows: ANP forward: 5′-CTAGACCACCTGG AGGAGAA-3′, ANP reverse: 5′-CAAGAGGGCAGATCTATC GG-3′, NGAL forward: 5′-CATTGGTCGGTGGGAACAG-3′, NGAL reverse: 5′-GATTCGTCAGCTTTGCCAAGT-3′, MMP-9 forward: 5′-GCCTTGGGTCAGGTTTAG-3′, MMP-9 reverse: 5′-GGCTTAGATCATTCTTCAGTG-3′, Egr-1 forward: 5′-CA AAGTGTTGCCACTGTTGGGTG-3′, Egr-1 reverse: 5′-CAA AGTGTTGCCACTGTTGGGTG-3′, IL-1β forward: 5′-CACG ATGCACCTGTACGATCA-3′ and IL-1β reverse: 5′-GTTGC TCCATATCCTGTCCCT-3′.

Western blot analysis

Western blot analysis was performed using polyclonal rabbit anti-mouse lipocalin-2/NGAL antibody (R&D Systems Inc., Minneapolis, MN) or monoclonal anti-human Egr-1 antibody (Abcam, Cambridge, UK) as the primary antibodies. Signals were detected using the enhanced chemiluminescence detection method (Amersham, the Netherlands) and quantified by densitometry. The amounts of NGAL and Egr-1 proteins were expressed relative to GAPDH.

Gelatin zymography

Electrophoresis was performed with conditioned medium collected from cultured cardiomyocytes or non-cardiomyocytes on a 10% polyacrylamide/SDS gel containing 1 mg/ml gelatin. SDS was removed by washing in 2.5% Triton X-100 for 1 hour at room temperature before the enzyme reaction. The gel was incubated overnight at 37 °C in enzyme buffer containing 50 mM Tris, pH 7.5, 200 mM NaCl, 5 mM CaCl2 and 0.02% Brij-35. Areas of gelatin degradation, identified as MMP activity, appeared as distinct white bands after staining the gels with 0.5% Brilliant Blue G-250.

Immunoprecipitation

For immunoprecipitation, conditioned medium of the cardiomyocytes isolated from volume-overloaded hearts or sham controls was incubated with protein G agarose (Sigma-Aldrich, St. Louis, MO) and polyclonal rabbit anti-mouse lipocalin-2/NGAL antibody (R&D Systems Inc., Minneapolis, MN), rabbit monoclonal anti-mouse MMP-9 antibody (Abcam Inc., Cambridge, MA), or a non-immuno serum as a control for 60 minutes. The protein G agarose beads were then thoroughly washed, and proteins were eluted with elution buffer. The eluted medium then underwent gelatin zymography analysis.

Enzyme-linked immunoassay

Blood samples were collected, centrifuged at 1,500 rpm for 5 minutes and stored at -80 °C until assay. Plasma NGAL and creatinine levels were measured in triplicate and duplicate, respectively, using an Enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, UK) according to the manufacturer's instructions.

Immunofluorescence staining

The frozen sections (8 μm thick) of the IVC specimens were blocked in 0.5% bovine serum albumin for 15 minutes, and then incubated with mouse monoclonal anti-α-cardiac actin (Sigma-Aldrich, St. Louis, MO), mouse monoclonal anti-porcine vimentin (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse monoclonal anti-rat CD31 (Santa Cruz Biotechnology, Santa Cruz, CA) at 37 °C for 1 hour. Samples were then treated with FITC-conjugated secondary antibody (1:500) at room temperature for 1 hour. The sections were then washed copiously with phosphate-buffered saline and exposed to goat anti-mouse NGAL (R&D Systems Inc, Minneapolis, MN) for 1 hour. The corresponding secondary antibody conjugated to Cy3 was applied. The specimens were then examined using a confocal laser scanning microscope (Leica TCS SP8X; Stansted, UK). The images were captured at a magnification of 400X. Each recorded image was 1024 × 1024 pixels in size, and the projection views of consecutive optical sections were captured at 0.5 μm. All specimens were examined within 24 hours of immunolabeling.

Statistical analysis

The data were described as mean and SD. Differences between two groups were determined using the unpaired t test. For multiple comparisons among groups, one-way ANOVA with post-hoc Scheffe test was used. A p value of < 0.05 was considered to be statistically significant.

A study flow chart is shown in Supplementary Figure 1.

RESULTS

Characterization of the rat model of chronic VO resulting from ACF

Chronic VO resulting from ACF was characterized using echocardiographic and hemodynamic studies as demonstrated in Figure 1. Chronic VO significantly increased the ratio of left ventricular (LV) end-diastolic dimension to posterior wall thickness (LVEDD/PW thickness) at 16 weeks but not at 8 weeks, and significantly decreased LV fractional shortening (LVFS) at 8 and 16 weeks compared with the corresponding sham controls. The LV end-diastolic pressure (LVEDP) significantly increased, and $+dP/dt_{max}$ and $-dP/dt_{max}$ significantly decreased at 16 weeks but not at 8 weeks. Correction of VO by closure of the ACF at 8 weeks after its initial creation decreased the LVEDD/PW thickness and increased the LVFS to levels similar to those of the corresponding sham controls at 4 weeks after correction. If correction of VO was performed at 16 weeks after the creation of ACF, the LVEDP remained significantly higher and both $+dP/dt_{max}$ and $-dP/dt_{max}$ remained significantly lower than the corresponding controls. Histological examination of the myocardium of left ventricular free wall stained with hematoxylin and eosin showed that there were no remarkable changes at 3 days compared with the sham controls. At 8 weeks, there was no significant increase in the size of cardiomyocytes as determined by the cross-sectional area. At 16 weeks of VO, the size of muscle fibers was observed. The endomysial and perimysial spaces were found to be enlarged. Correction of VO at this time point resulted in narrowing of the enlarged endomysial and perimysial spaces without obvious changes in the size of muscle fibers (Figure 2). Taken together, these findings indicated that the ACF rats remained at the compensatory phase at 8 weeks (VO_{comp}) and progressed to the HF phase at 16 weeks (VO_{HF}), which is consistent with the findings of a previous study.[18] In addition, cor-

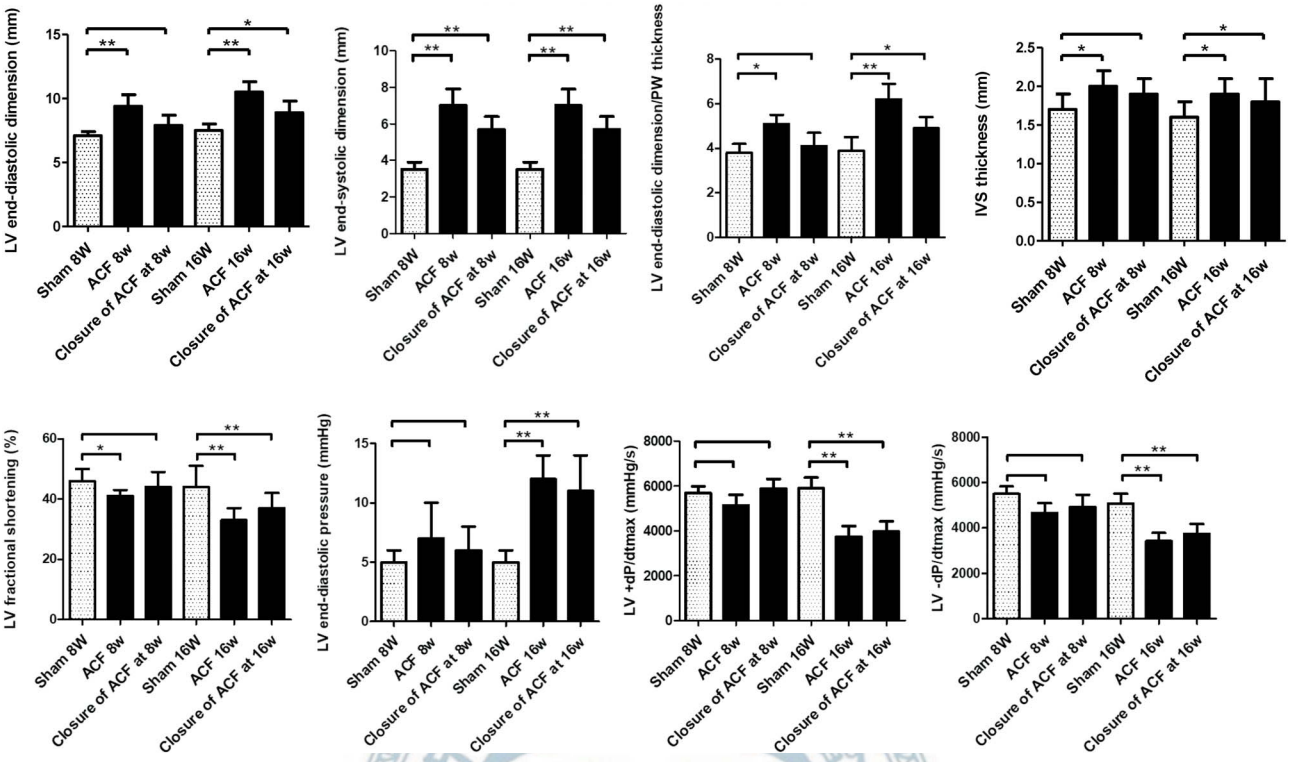

Figure 1. *Echocardiography and hemodynamic assessment of rats with aortocaval fistula (ACF) at 8 and 16 weeks and rats with closure of ACF at 8 or 16 weeks (n = 5 for group). Values are means ± SD. IVS, interventricular septum; LV, left ventricular; PW, posterior wall. * p < 0.05 and ** p < 0.01 vs. corresponding sham rats.*

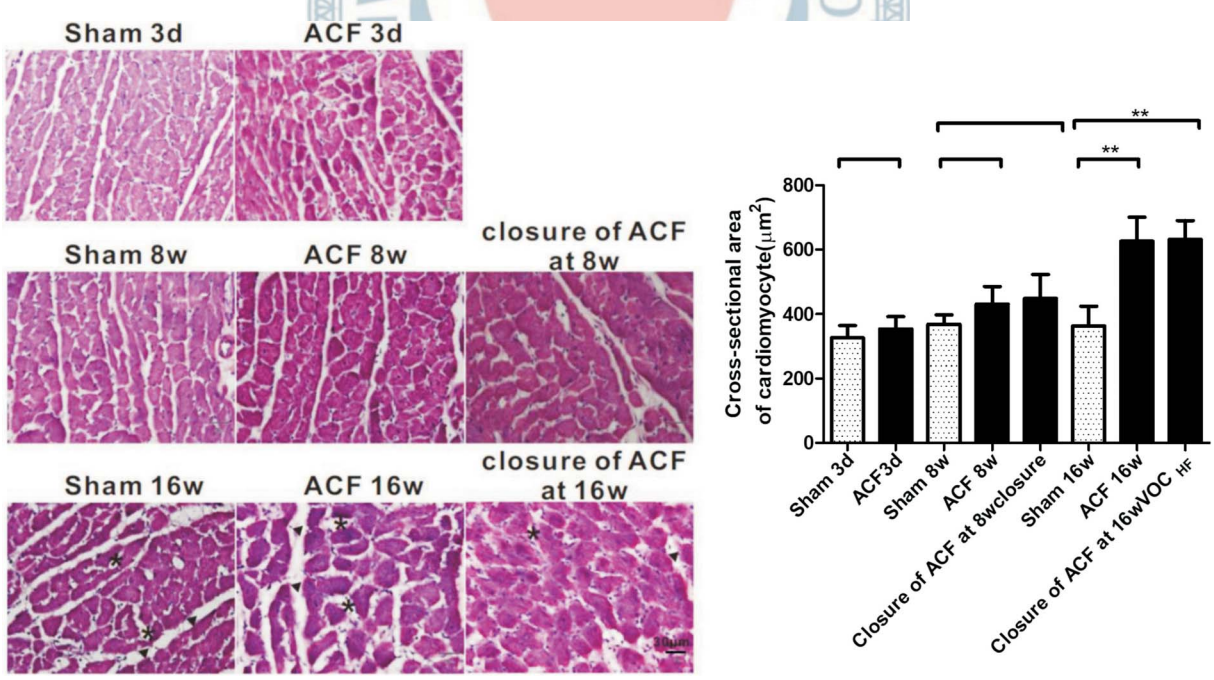

Figure 2. *Histological examination of the myocardium of rats with aortocaval fistula (ACF) at 3 days, 8 and 16 weeks and the myocardium of rats with ACF surgically closed at 8 or 16 weeks after initial creation (n = 4 for each group). (A) Sections of myocardium of left ventricular free wall was stained with hematoxylin and eosin. Enlargement of endomysial spaces (asterisks) and perimysial spaces (closed triangles) were observed in the myocardium of ACF rats at 16 weeks. (B) Comparison of the size of muscle fibers determined by the cross-sectional area. * p < 0.05 and ** p < 0.01.*

rection of VO at 8 weeks (VOC_{comp}) reversed dilation of the LV with LV function remaining normal, whereas delayed correction of VO at 16 weeks (VOC_{HF}) did not reverse the LV dysfunction. Plasma creatinine levels in the ACF rats at different phases were determined by ELISA. The creatinine levels in the experimental rats at all phases did not differ from those of their corresponding sham controls, except in the ACF rats with delayed correction of VO. The findings of echocardiographic and hemodynamic studies and plasma creatinine levels are summarized in Table 1.

Myocardial expression of NGAL during the development of HF

We first examined the gene expression of NGAL in the LV exposed to acute VO using RT-qPCR. The gene expression of NGAL increased by 28 folds at day 3, and then decreased gradually but remained significantly higher than the controls at 2 weeks (Figure 3A). On exposure to chronic VO, the expression of NGAL significantly increased at both transcriptional and translational levels at the compensatory phase. When progressing to the HF phase, the expression remained higher than the sham controls. The expression level was not different between both phases. When the VO was surgically corrected at the compensatory phase, the expression was reversed to the level of the sham controls. In contrast, correction of VO in the HF phase did not reverse the expression level, and the level was not significantly different from that of the HF phase (Figure 3B and 3C). These findings demonstrated that myocardial NGAL expression was upregulated during the development of HF. There was no significant change in expression level during progression from the compensatory phase to the HF phase.

To identify the cell types responsible for the increase in myocardial NGAL expression, we isolated cardiomyocytes, fibroblasts and endothelial cells from VO LV and sham controls at 3 days. Real-time PCR analysis showed that NGAL was upregulated in cardiomyocytes but not in fibroblasts or endothelial cells (Figure 3D). Double immunohistochemistry staining using anti-sarcolemma-actin, anti-CD31 and anti-vimentin antibody as the markers of cardiomyocytes, endothelial cells and fibroblasts, respectively, showed colocalization of anti-NGAL with anti-sarcolemma-actin signals but not with anti-CD31 or fibroblasts (Figure 3E), confirming that cardiomyocytes were the myocardium cell type responsible for the upregulation of NGAL.

Renal expression of NGAL during the development of HF

In exposure to acute VO, transcription of NGAL in the kidney did not change at day 3, week 1 or 2 compared with baseline (Figure 4A). When chronically exposed to VO, the renal expression of NGAL did not significantly change at the transcriptional and translational level in either compensatory phase or HF phases. Correction of VO in the compensatory phase did not alter the expression. In contrast, correction of VO in the HF phase induced markedly increased gene and protein expressions of NGAL (Figure 4B and 4C). Since the renal

Table 1. Cardiac remodeling, hemodynamics and plasma creatinine levels of rats with aortocaval fistula

	Sham 8 w	ACF 8 w	4 w after close of ACF at 8 w	Sham 16 w	ACF 16 w	4 w after close of ACF at 16 w
LV end-diastolic dimension, mm	7.1 ± 0.3	9.4 ± 0.9*	7.9 ± 0.8	7.5 ± 0.5	10.5 ± 0.8*	8.9 ± 0.9*
LV end-systolic dimension, mm	3.4 ± 0.4	5.5 ± 0.5*	4.4 ± 0.6*	3.5 ± 0.4	7.0 ± 0.9#	5.7 ± 0.7*
IVS thickness, mm	1.7 ± 0.2	2.0 ± 0.2*	1.9 ± 0.2*	1.6 ± 0.2	1.9 ± 0.2*	1.8 ± 0.3*
LV end-diastolic dimension/PW thickness	3.6 ± 0.4	5.4 ± 0.4#	4.1 ± 0.6	3.9 ± 0.6	6.2 ± 0.7#	4.9 ± 0.5*
LV fractional shortening, %	46 ± 4	41 ± 2	44 ± 5	44 ± 7	33 ± 4*	37 ± 5*
LV end-diastolic pressure, mmHg	5 ± 1	7 ± 4	6 ± 3	5 ± 1	12 ± 2#	11 ± 3#
LV +dP/dtmax, mmHg/s	5684 ± 298	4737 ± 476	5883 ± 433	5899 ± 476	3737 ± 476*	3972 ± 452*
LV -dP/dtmax, mmHg/s	-5504 ± 324	-4435 ± 438	-4924 ± 542	-5074 ± 432	-3422 ± 358*	-3744 ± 432*
Plasma creatinine (mg/dL)	0.7 ± 0.2	0.6 ± 0.3	0.8 ± 0.3	0.8 ± 0.3	0.8 ± 0.2	1.0 ± 0.4*

Values are means ± SD.

ACF, aortocaval fistula; IVS, interventricular septum; LV, left ventricle; PW, posterior wall; SD, standard deviation.

* $p < 0.05$ and # $p < 0.01$ vs. corresponding sham.

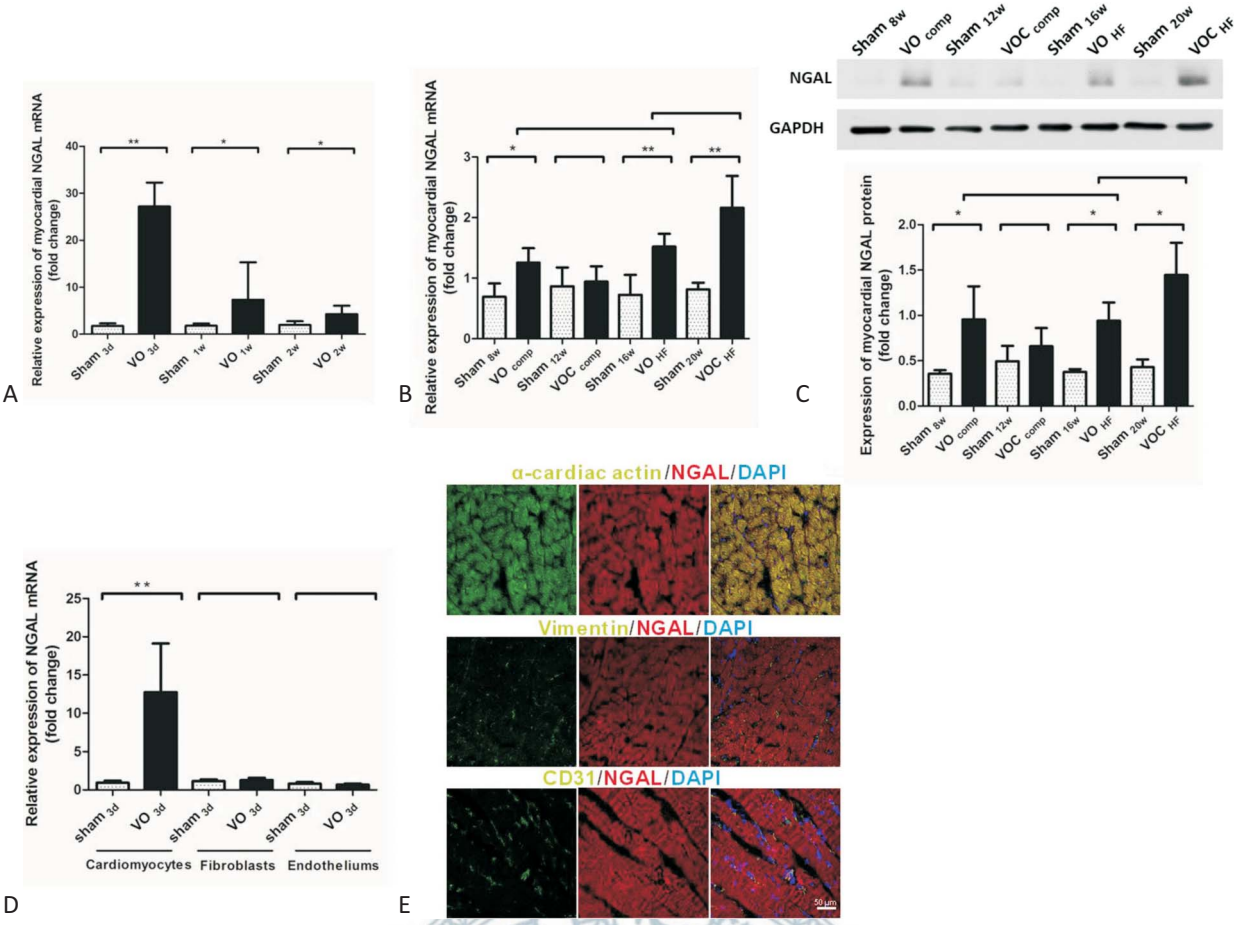

Figure 3. *Neutrophil gelatinase-associated lipocalin (NGAL) expression in the heart of rat with volume-overload (VO). (A) Myocardial NGAL gene expressions in rats with acute VO at 3 days, 1 and 2 weeks (n = 4 for each group). (B and C) Myocardial NGAL gene (B) and protein (C) expression in rats with chronic VO in the compensatory phase (VO_{comp}) or heart failure phase (VO_{HF}), or after correction of VO in the compensatory (VOC_{comp}) or the heart failure phase (VOC_{HF}) (n = 5 for each group). (D) NGAL gene expression in cardiomyocytes, fibroblasts and endothelial cells isolated from left ventricle of rat with VO for 3 days (n = 4 for each group). (E) Immunofluorescence study using anti-α-cardiac actin, anti-vimentin or anti-CD31 antibodies as cell markers for cardiomyocytes, fibroblast or endothelial cells (green) to identify the cell types of myocardium responsible for expression of NGAL (red) (n = 3). * $p < 0.05$ and ** $p < 0.01$.*

NGAL expression was upregulated and the plasma creatinine level was mildly but significantly increased after correction of VO in the HF phase, we examined kidney sections to confirm the presence of kidney injury in this phase. Tissue sections of the kidney stained with hematoxylin and eosin demonstrated dilation of tubules and formation of casts with intratubular obstruction, indicating acute tubular necrosis (Figure 4D). These findings indicated that the renal NGAL expression could sensitively reflect renal injury as shown in previous studies.[2-6]

Circulating levels of NGAL in the rats with acute VO, chronic VO or with correction of chronic VO

We then analyzed the plasma levels of NGAL in the ACF rats in different phases using ELISA. In exposure to acute VO, the plasma NGAL level was highly significantly increased compared with that at baseline, and then decreased progressively at 1 and 2 weeks although remaining significantly higher compared with baseline. Since the expression of renal NGAL did not significantly change and the temporal pattern of myocardial NGAL expression was consistent with that of plasma NGAL level, the myocardium was likely to be the origin of increased plasma NGAL in the acute phase of VO although the potential contribution of vascular injury could not be excluded (Figure 5A). When chronically exposed to VO, the plasma NGAL level increased in both the compensatory and HF phases compared with the corresponding sham con-

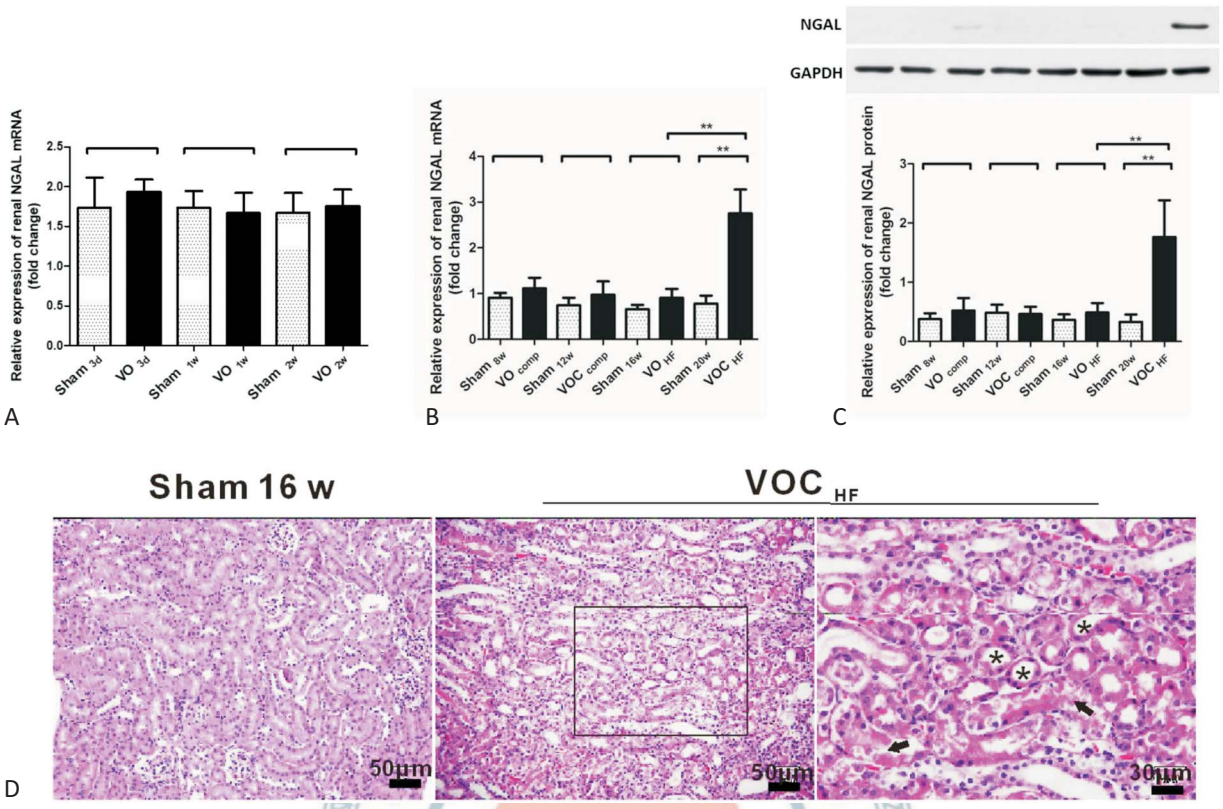

Figure 4. *Neutrophil gelatinase-associated lipocalin (NGAL) expression in the kidney of rat with volume-overload (VO). (A) Renal NGAL gene expressions in rats with acute VO at 3 days, 1 and 2 weeks (n = 4 for each group). (B and C) Renal NGAL gene (B) and protein (C) expression in rats with chronic VO in the compensatory phase (VO_{comp}) or heart failure phase (VO_{HF}), or after correction of VO in the compensatory (VOC_{comp}) or the heart failure phase (VOC_{HF}) (n = 5 for each group). (D) Hematoxylin and eosin staining for section of kidney of sham rats or rats in the VOC HF phase (n = 4 for each group). Tubular dilation (asterisks) and cast formation with intratubular obstruction (arrows) was found in the VOC HF phase. * p < 0.05 and ** p < 0.01.*

trols. When comparing the plasma NGAL levels of both phases, the level did not significantly differ. Correction of VO in the compensatory phase reversed the plasma NGAL level to the that of the corresponding sham controls. Interestingly, correction of VO in the HF phase resulted in a marked increase in the plasma NGAL level to about 4 folds that of the sham controls and about 3 folds that of the HF phase (Figure 5B). Since the renal NGAL expression was markedly upregulated and the myocardial NGAL expression was not significantly increased in this phase compared with the HF phase, it is plausible that this high increase in plasma NGAL level was attributable to the highly upregulated renal NGAL expression. The findings of the analysis of plasma NGAL level indicated that plasma NGAL level could sensitively reflect the changes in renal function, since the mild but significant increase in plasma creatinine level found after correction of VO in the HF phase was associated with

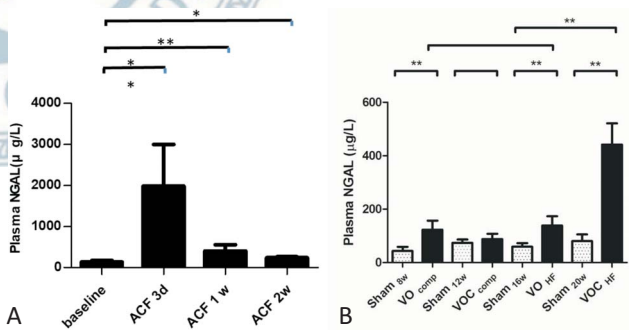

Figure 5. *Plasma levels of neutrophil gelatinase-associated lipocalin (NGAL) in rats with acute or chronic volume-overload (VO). (A) Plasm NGAL level was measured by enzyme-linked immunosorbent assay (ELISA) at day 3, 1 week or 2 weeks after creation of aortocaval fistula. (B) Plasm NGAL level was measured in the compensatory phase (VO_{comp}) or the heart failure phase (VO_{HF}), or after correction of VO in the compensatory phase (VOC_{comp}) or the heart failure phase (VOC_{HF}) (n = 6). * p < 0.05 and **p < 0.01.*

the high increase in plasma NGAL level. In contrast, the plasma NGAL level did not seem to be an indicator of

the severity of LV dysfunction, since the significantly worse LV function in the HF phase and after correction of VO in the HF phase compared with the compensatory phase was not associated with an increase in plasma NGAL level.

MMP-9/NGAL complex in volume-overloaded hearts

We next investigated whether NGAL upregulation was associated with formation of the MMP-9/NGAL complex in exposure to VO. We collected the conditioned medium of cultured cardiomyocytes and fibroblasts isolated from volume-overloaded rats or sham controls at 3 days and examined the presence of MMP-9/NGAL complex using gelatin zymography. In the mediums of cardiomyocytes, a double band at 95 kDa, reflecting the latent and active forms of MMP-9 was detected in both groups, with higher enzymatic activity in the VO group. In the medium of the VO group, an additional double band was detected at 125 kDa, indicating the MMP-9/NGAL complex based on the known molecular weight. The medium of cultured fibroblasts was used as a negative control, since upregulation of NGAL was not found in fibroblasts isolated from the volume-overloaded hearts. A double band at 95 kDa and a single band at 70 kDa were detected in both sham control and VO groups, reflecting the latent and active forms of MMP-9 and MMP-2, respectively. The enzymatic activities were similar between both groups. In contrast to the mediums of cardiomyocytes, no zymolytic band at 125 kDa was found in medium of fibroblasts of either group (Figure 6A).

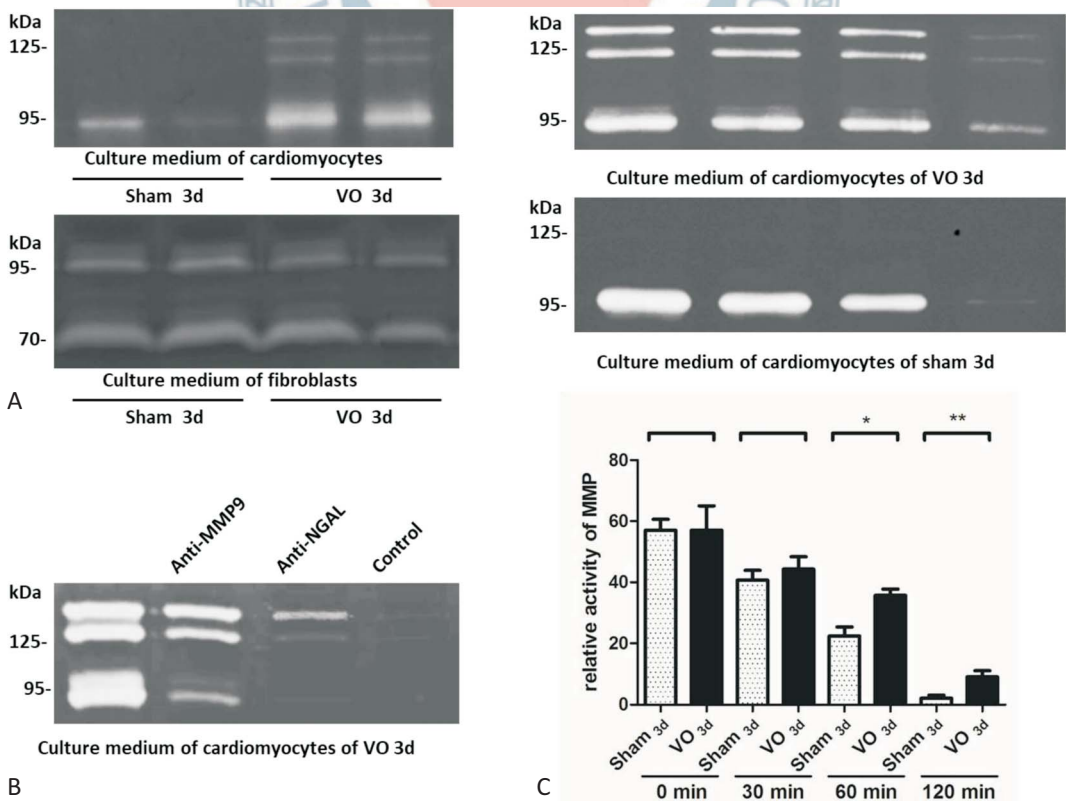

Figure 6. *Presence of MMP-9/NGAL complex in conditioned mediums of cardiomyocytes isolated from heart with VO for 3 days (n = 4). (A) Representative gelatin zymography of cultured medium of cardiomyocytes and fibroblasts isolated from volume-overloaded heart or control. In mediums of cardiomyocytes, a double band at ≒ 95 kDa was detected in both VO and control groups. An additional double band was detected at 125 kDa in the VO group but not in the control group. In mediums of fibroblasts, the double band at 125 kDa was not detected in either group. (B) Immunoprecipitation for mediums of the VO group using anti-NGAL, anti-MMP-9 or non-immune control antibodies. The elution fractions were subjected to gelatin zymography analysis. The anti-MMP-9 antibody immunoprecipitated activities at both 95 and 125 kDa; and the anti-NGAL antibody immunoprecipitated activity at 125 kDa only. (C) MMP-9/NGAL complex on protects MMP-9 from decay of enzyme activity. Mediums of cardiomyocytes of VO and control groups were titrated to achieve a similar total enzymatic activity determined by densitometry analysis of gelatin zymography and incubated at 37° for 30, 60 and 120 minutes. Enzyme activity remained after incubation was assayed by zymography and measured by scanning densitometry (n = 4). * $p < 0.05$ and ** $p < 0.01$. MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; VO, volume overload.*

We then verified the identity of gelatinolytic activity at 125 kDa by immunoprecipitation. Both anti-MMP-9 and anti-NGAL antibodies immunoprecipitated the gelatinolytic activity at 125 kDa, which confirmed that this gelatinolytic activity reflected the MMP-9/NGAL complex (Figure 6B). Taken together, these findings showed that the upregulation of NGAL in the cardiomyocytes of volume-overloaded hearts was associated with formation of the MMP-9/NGAL complex. The absence of a zymolytic band at 125 kDa in the medium of cultured fibroblasts served as a negative control.

We then tested whether the MMP-9/NGAL complex affected MMP-9 activity. The 10× concentrated medium of the cultured cardiomyocytes isolated from both the VO and sham control groups was titrated to give a similar total MMP-9 activity as determined by zymographic analysis and densitometry. The medium was then incubated at 37 °C to allow for degradation of MMP-9 activity. The MMP-9 activity decreased gradually in both groups; however, the degradation was significantly slower in the VO group with the total remaining MMP-9 activity significantly higher at 60 and 120 minutes compared with the control group (Figure 6C). These findings confirmed that the presence of the MMP-9/NGAL complex in the volume-overloaded hearts protected MMP-9 from degradation.

Egr-1 regulated NGAL expression in cardiomyocytes through the IL-1 pathway

Plasma NGAL has been shown to be strongly associated with inflammation in the general population.[13] Plasma NGAL level has also been shown to be correlated with the level of high-sensitivity C-reactive protein in patients with chronic HF of ischemic cause.[10] Egr-1 is a master transcription factor that regulates inflammatory responses,[16] and it has been shown to play an important role in mediating myocardial inflammation.[19,20] Therefore, we tested whether Egr-1 regulates the expression of myocardial NGAL. Western blot analysis showed that Egr-1 was upregulated in the volume-overloaded hearts at both compensatory and HF phases compared with their corresponding sham controls (Figure 7A). In neonatal cardiomyocytes, transient transfection with Egr-1 induced upregulation of the mRNA of NGAL, MMP-9 and interleukin (IL)-1β by approximately 25, 35 and 30 folds, respectively (Figure 7B). Furthermore, Egr-1-induced up-

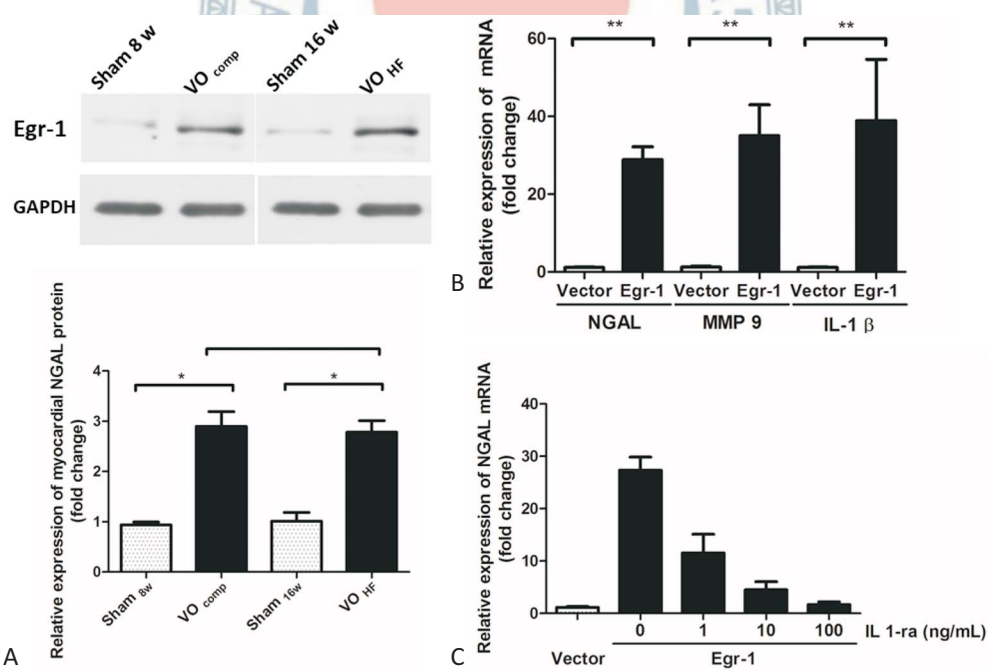

Figure 7. *Early growth response (Egr)-1 regulating expressions of NGAL, MMP-9 and IL-1β in cardiomyocytes. (A) Representative Western blots for Egr-1 protein in chronic volume-overloaded hearts in the compensatory phase (VO_{comp}) or heart failure phase (VO_{HF}) (n = 3). (B) Gene expressions of NGAL, MMP-9 and IL-1 in neonatal cardiomyocytes transiently transfected with Egr-1. (C) NGAL gene expression in neonatal cardiomyocytes transiently transfected with Egr-1 and treated with rat IL-1 receptor antagonist at the dose of 0, 1, 10 and 100 ng/mL. * p < 0.05 and ** p < 0.01. Egr, early growth response; IL, Interleukin; MMP, matrix metalloproteinase; NGAL, neutrophil gelatinase-associated lipocalin; VO, volume overload.*

regulation of NGAL in neonatal cardiomyocytes was dose-dependently suppressed by an IL-1 receptor antagonist (Figure 7C). These findings indicated that Egr-1 mediates the expression of NGAL in cardiomyocytes through the IL-1 pathway.

DISCUSSION

In the present study, we found that acute VO strongly induced the upregulation of myocardial NGAL. During the development of HF induced by chronic VO, NGAL expression was significantly upregulated in the heart but not in the kidney. The myocardial expression of NGAL was upregulated in both the compensatory and HF phases, and the expression level was not significantly different between both phases. Correction of VO in the compensatory phase reversed the upregulation of myocardial NGAL. In contrast, correction of VO in the HF phase did not result in a significant change in myocardial NGAL expression. Interestingly, correction in the HF phase strongly induced the upregulation of renal NGAL. These findings showed that the changes in myocardial NGAL expression during the development of HF were not consistent with the severity of LV dysfunction, since the expression level was not significantly different even though LV function differed significantly among the compensatory and HF phases and after correction of VO in the HF phase.

On the other hand, consistent with previous studies,[2-6] we demonstrated that renal NGAL expression could sensitively reflect renal injury, since a mild but significant increase in plasma creatinine level was associated with a highly increased expression of renal NGAL after correction of VO in the HF phase. The mechanism of renal NGAL upregulation is beyond the scope of this study. Kidney injury resulting from hemodynamic changes and adaptation of the neurohormonal system after delayed correction of VO may contribute to the upregulation of NGAL.

In patients with HF, the potential role of circulating NGAL level as a predictor of clinical outcomes in terms of cardiac mortality or worsening of renal function has been tested in numerous studies, however the results have been inconsistent.[7-14] In this study, the rat model of HF induced by chronic VO is likely to represent a model of chronic stable HF. We found that the plasma NGAL levels were significantly increased in both the compensatory and HF phases. However, the level was not significantly higher in the HF phase, a pattern that is consistent with the pattern of myocardial NGAL expression. These findings suggest that plasma NGAL level is not an effective indicator to differentiate the severity of LV dysfunction during the development of HF, and that it may not be an effective outcome predictor of HF. On the other hand, the mildly but significantly increased plasma creatinine level found after delayed correction of VO in the HF phase was shown to be associated with a marked elevation in plasma NGAL level, indicating that circulating NGAL level is a sensitive marker of renal injury, as shown in previous studies.[2-6] These findings are consistent with a previous clinical study reported by Shrestha et al.[14] In 130 patients with chronic systolic HF, they found that systemic NGAL levels were largely determined by underlying impairment of renal function rather than myocardial function. In elderly patients with chronic ischemic HF, Nymo et al. also showed that serum NGAL level remained a significant predictor of adverse outcomes after adjusted for demographic and clinical variables. However, this association was lost after adjusting for apolipoprotein A-1 (ApoA-1), estimated glomerular filtration rate (eGFR), hs-CRP, and NT-proBNP.[10]

In this study, we demonstrated that the presence of the MMP-9/NGAL complex in volume-overloaded hearts enhanced the enzymatic activity of MMP-9, as reported in various cancers and inflammatory diseases.[21,22] MMP-9 has been shown to play an important role in cardiac remodeling through modification of the extracellular matrix.[23,24] Persistent upregulation of myocardial NGAL during the development of HF supports that NGAL may be actively involved in remodeling of the heart through modification of the extracellular matrix. Therefore, NGAL may be a potential therapeutic target to prevent adverse cardiac remodeling. In addition to enhancing MMP-9 activity, NGAL was recently identified to be a downstream mineralocorticoid receptor target. NGAL knockout mice were shown to have lower cardiac interstitial fibrosis and inflammation, and better cardiac function after myocardial infarction.[25] On the other hand, NGAL secreted by neutrophils in infarcted myocardium reported to be favorable for cardiac remodeling by skewing macrophages towards a phenotype with high capacity to engulf apoptotic cells and consequently regulate

fibrosis.[26] These findings indicate that NGAL plays pleiotropic roles in the pathogenesis of HF.

In this study, we found that Egr-1 was upregulated during the development of HF induced by VO, and that the expression of NGAL in cardiomyocytes was regulated by Egr-1 through the IL-1 pathway. Interestingly, we found that Egr-1 overexpression in cardiomyocytes also induced the expression of MMP-9. The simultaneous upregulation of both NGAL and MMP-9 may further enhance MMP-9 activity through formation of the MMP-9/NGAL complex as shown in this study. Egr-1 is emerging as a key player in myocardial injury through inflammation.[19,20] Our findings suggest that NGAL may be involved in the myocardial injury orchestrated by Egr-1.

Limitations

This study has several limitations. We focused on the heart failure model resulting from VO, and the pattern of cardiac and renal NGAL expressions and the circulating level of NGAL may be different in HF of different etiologies. In addition, we did not examine urine NGAL level. In patients with chronic HF, urine NGAL levels at admission have been associated with an increased risk of subsequent worsening of renal function.[27] Moreover, urine NGAL has been associated with a primary endpoint of all-cause mortality and HF hospitalization in chronic HF patients.[28] The association between changes in urine NGAL levels and severity of cardiac dysfunction during the development of HF remain unclear.

CONCLUSIONS

During the development of HF resulting from VO, NGAL expression was upregulated in the heart but remained unchanged in the kidney. Circulating NGAL levels could not differentiate the severity of cardiac dysfunction but could sensitively reflect kidney injury in chronic stable HF. Upregulation of NGAL was associated with formation of the NGAL/MMP-9 complex, which enhanced the enzymatic activity of MMP-9. Furthermore, myocardial NGAL was upregulated by Egr-1 through the IL-1 pathway. Therefore, NGAL may be a potential therapeutic target for adverse cardiac remodeling of volume-overloaded hearts although it may not be an effective outcome predictor in chronic stable HF.

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DECLARATION OF CONFLICT OF INTEREST

All the authors declare no conflict of interest.

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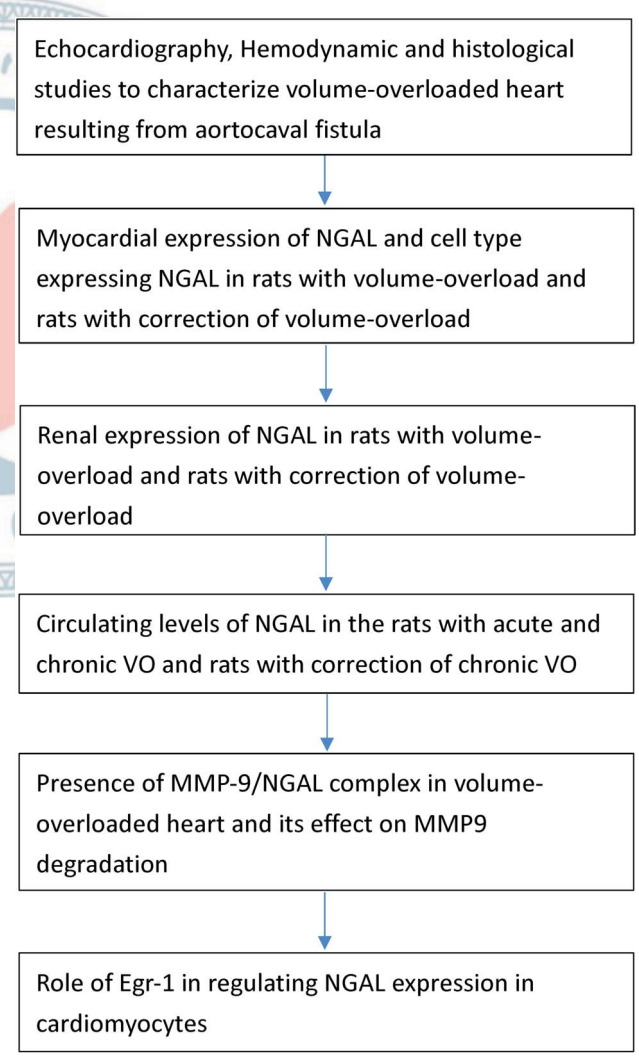
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. *Flow chart of experiment.*