Original Article

Excess Aldosterone under Normal Salt Diet Induces Cardiac Hypertrophy and Infiltration *via* Oxidative Stress

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Aldosterone is known to play a role in the pathophysiology of some cardiovascular diseases. However, previous studies on aldosterone infusion have been mostly performed in animals receiving sodium loading and uninephrectomy, and thus the cardiac action of aldosterone alone remains to be fully clarified. The present study was undertaken to investigate the direct cardiac action of aldosterone infusion alone in rats not subjected to salt loading and uninephrectomy. Aldosterone (0.75 μg/h) was subcutaneously infused into rats *via* an osmotic minipump for 14 days. Aldosterone infusion, under a normal salt diet, induced only a slight increase in the blood pressure of normal rats throughout the infusion. However, aldosterone significantly induced cardiac hypertrophy, as shown by echocardiography and measurement of cardiomyocyte cross-sectional area. Furthermore, aldosterone caused not only cardiac interstitial macrophage infiltration but also cardiac focal inflammatory lesions, which were associated with an increase in cardiac monocyte chemoattractant protein-1 (MCP-1) and osteopontin mRNA. The slight elevation of blood pressure by aldosterone infusion was completely prevented by tempol, the superoxide dismutase mimetic. However, tempol failed to suppress cardiac hypertrophy, the formation of inflammatory lesions, and upregulation of cardiac MCP-1 and osteopontin by aldosterone, while *N*-acetylcysteine could inhibit all of them. Our data provide evidence that aldosterone alone can induce cardiac hypertrophy and severe inflammatory response in the heart, independently of blood pressure, even in the absence of salt loading or nephrectomy. Aldosterone seems to induce cardiac inflammation and gene expression *via* oxidative stress that is inhibited by *N*-acetylcysteine but not by tempol. (*Hypertens Res* 2005; 28: 447–455)

***Key Words*: aldosterone, angiotensin II, cardiac hypertrophy, oxidative stress, inflammation**

Introduction

Accumulating clinical and experimental evidence shows that aldosterone (ALD) is directly implicated in the pathophysiology of cardiovascular diseases as well as hypertension (*1–11*). Excess ALD in patients with primary aldosteronism is associated with left ventricular (LV) hypertrophy and alter-

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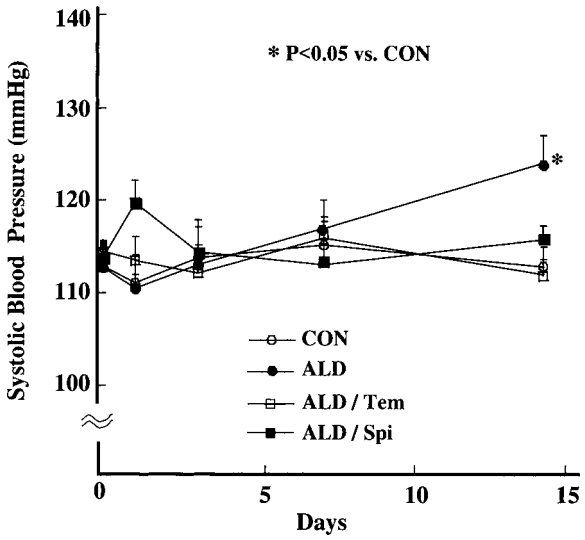

Fig. 1. *Time course of systolic blood pressure (SBP) in ALD-infused rats. CON, control; ALD, aldosterone; ALD/Tem, ALD infusion with tempol treatment; ALD/Spi, ALD infusion with spironolactone treatment.*

ations of myocardial textures (*12–14*). Blockade of the mineralcorticoid receptor with spironolactone has been shown to strikingly improve outcome, LV hypertrophy and fibrosis in patients with heart failure (*6*). Treatment with eplerenone, a newly developed specific ALD blocker, reduces cardiovascular mobility and mortality among patients with acute myocardial infarction complicated by LV dysfunction and heart failure (*5*).

The combined administration of ALD and salt in uninephrectomized rats has been shown to induce the development of the coronary inflammatory phenotype in the rat heart with no evidence of significant myocardial fibrosis (*7–9, 15*). Cardiac inflammatory changes in ALD-infused and salt-loaded uninephrectomized rats are mediated by oxidative stress (*9*). Thus, experimental findings indicate that the cardiac inflammation induced by ALD might contribute to cardiac injury. However, most previous studies on cardiac injury in the ALD infusion model have been performed on animals with uninephrectomy, excess salt loading, or both. Little information is available concerning the effect of excess ALD on cardiac injury of rats not subjected to salt loading and nephrectomy. Therefore, it is unclear whether the cardiac injury induced by ALD infusion in previous studies is due to the direct action of ALD itself or the sodium loading or uninephrectomy. In the present study, to examine this open question, we examined the cardiac effects of ALD infusion alone on normal rats not subjected to uninephrectomy or salt loading. Our results provide evidence that excess ALD alone, without salt loading or uninephrectomy, directly induces cardiac hypertrophy and focal inflammatory lesions through oxidative stress.

Methods

Experimental Protocol

All procedures were in accordance with the National Research Council's guidelines. Male Sprague-Dawley rats (Clea Japan, Tokyo, Japan) weighing 230 to 260 g were used in this study and fed a normal rat chow containing 0.5% NaCl (CE2; Clea Japan). ALD (0.75 μg/h) dissolved in 5% ethanol was continuously infused subcutaneously into rats *via* an osmotic minipump (ALZET, DURECT Co., Cupertino, USA) for 2 weeks.

In the first series of experiments, animals were divided into four groups ($n=6$ to 7 in each group): 1) a control group (CON; 5% ethanol infusion); 2) a group receiving ALD infusion; 3) a group receiving ALD infusion and spironolactone treatment (ALD receptor antagonist; 100 mg/kg/day); and 4) a group receiving ALD infusion and tempol (*16, 17*) (superoxide dismutase mimetic, 1 mmol/l in the drinking water). Spironolactone suspended in 0.5% carboxymethyl cellulose (CMC) was given to rats by gastric gavage once a day from 3 days before implantation of the osmotic minipump until the end of the experiments. Tempol (1 mmol/l) was given to rats in drinking water. The doses of ALD, spironolactone, and tempol were determined on the basis of the results from previous studies in rats (*8, 9, 18–20*). Systolic blood pressure (SBP) was periodically measured in each group of rats during ALD. After 14 days of the infusion, transthoracic echocardiographic studies were performed, and then blood samples were collected by puncturing the heart, and heart samples were excised for pathological examination and immunohistochemistry.

In the second series of experiments, four animal groups were studied ($n=5$ to 7 in each group): 1) a control (CON; 5% ethanol), 2) an ALD infusion (0.75 μg/h), 3) an ALD infusion (0.75 μg/h) and tempol (3 mmol/l in drinking water), 4) and an ALD infusion (0.75 μg/h) and *N*-acetylcysteine (NAC) (antioxidant; 200 mg/kg/day, i.p. daily) group. SBP was periodically measured by the tail-cuff method. After 14 days of the infusion, transthoracic echocardiography was performed, and then the heart was removed for pathological examination, immunohistochemistry and Northern blot analysis.

Blood Pressure Measurement

SBP and heart rate were measured in conscious rats by tail-cuff plethysmography (BP-98A; Softron Co., Tokyo, Japan).

Histological Examination

The hearts were fixed in 4% paraformaldehyde overnight. Then, they were embedded in paraffin, sectioned into 5-μm slices, and immunostained with the following primary antibodies: anti-ED-1 (for identification of monocytes/macrophages, working dilution 1:500), and CD45 (leukocyte

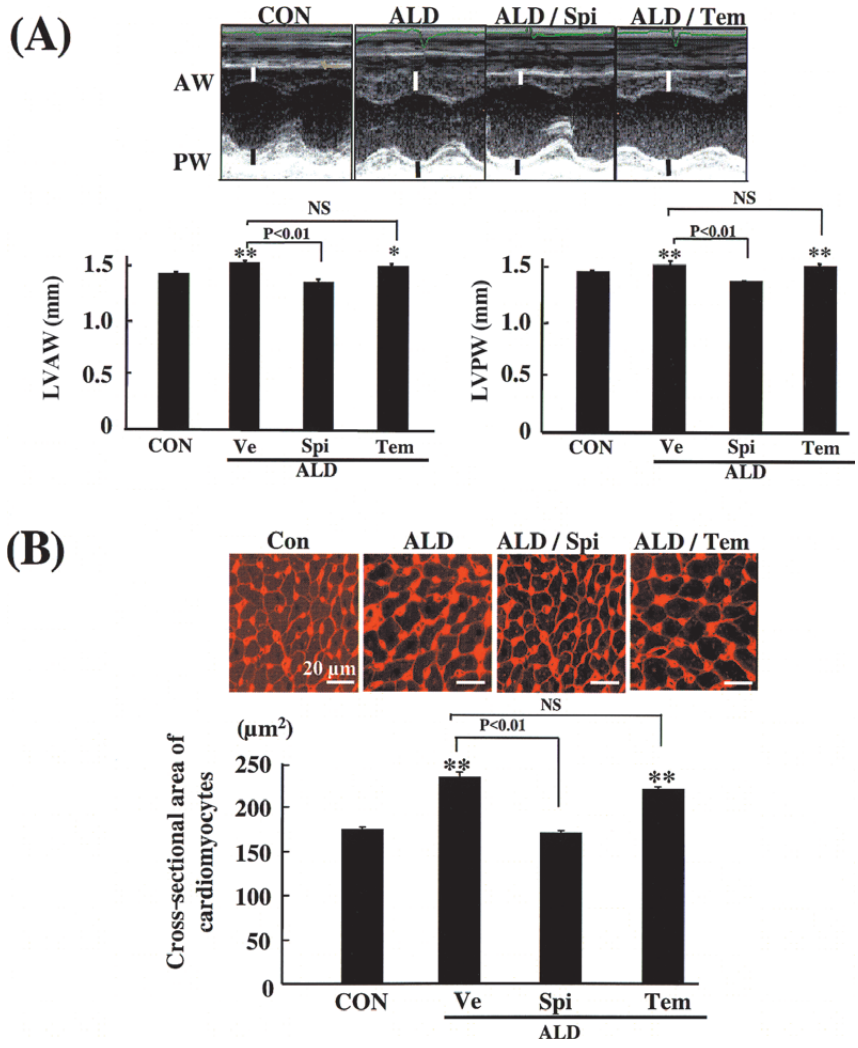

Fig. 2. *Echocardiographic analysis (A) and cardiomyocyte cross-sectional area (CSA) (B) of ALD-infused rats. A: The top panels show representative images of echocardiograph of the left ventricle (LV) after 2 weeks of treatment. AW, LV anterior wall thickness at end diastole; PW, LV posterior wall thickness at end diastole. Ve, vehicle; Spi, spironolactone; Tem, tempol. The white and black bars indicate AW and PW, respectively. Bar graph shows quantitative analysis of AW and PW thickness. Each bar represents the mean±SEM (*n = *6 to 7). *p < *0.05, **p < *0.01 vs. *the control group. NS, not significant. B: The top panels show representative images of fluorescence micrographs of LV cardiomyocyte CSA. Original magnification, ×400. The bar graph shows results of the quantitative analysis of LV cardiomyocyte CSA. The abbreviations are the same as in A. Each bar represents the mean±SEM (*n = *6 to 7 per group). **p < *0.01 vs. *the control group.*

common antigen, working dilution 1:100). Positive staining was detected using horseradish peroxidase-conjugated secondary antibodies (Nichirei, Tokyo, Japan) by incubating the sections with diaminobenzidine (DAKO, Carpinteria, USA). ED-1-positive focal inflammatory lesions were counted in 4 horizontal cardiac sections in individual rats; and the average number of focal inflammatory lesions per section was obtained in individual rats.

Measurement of cardiomyocyte cross-sectional area (CSA) was performed as previously described in detail (*21*). Approximately 100 cells were counted per sample and the average was used for analysis.

Northern Blot Analysis

All procedures were performed as described in detail in our previous reports. The densities of individual mRNA bands were measured by using a bioimaging analyzer (BAS-5500; Fuji Photo Film Co., Tokyo, Japan) (*22*).

Echocardiographic Assessment

Transthoracic echocardiographic studies were performed on the 14th day with an echocardiographic system equipped with 12-MHz echocardiographic probe (SONOS 5500; Agilent

Table 1. Echocardiographic Parameters after 2 Weeks of ALD Infusion

	CON (n=7)	ALD (n=7)	ALD/Spi (n=6)	ALD/Tem (n=7)
BW (g)	368±4	367±8	354±10	356±4
Echocardiography				
HR	228±9	214±12	222±11	227±6
LVEDD (mm)	8.4±0.3	8.5±0.2	8.6±0.4	9.0±0.1
LVESD (mm)	5.1±0.4	5.2±0.4	5.3±0.3	5.1±0.2
FS (%)	40.3±2.2	40±3.4	39.3±1.9	39±0.9
EF (%)	78.4±2.2	77.2±3.5	77.3±2.1	77.2±0.9

CON, control; ALD, aldosterone; ALD/Spi, ALD infusion with spironolactone treatment; ALD/Tem, ALD infusion with tempol treatment; BW, body weight; HR, heart rate; LVEDD, left ventricular (LV) end-diastolic dimension; LVESD, LV end-systolic dimension; FS, fractional shortening; EF, LV ejection fraction. Values are mean±SEM.

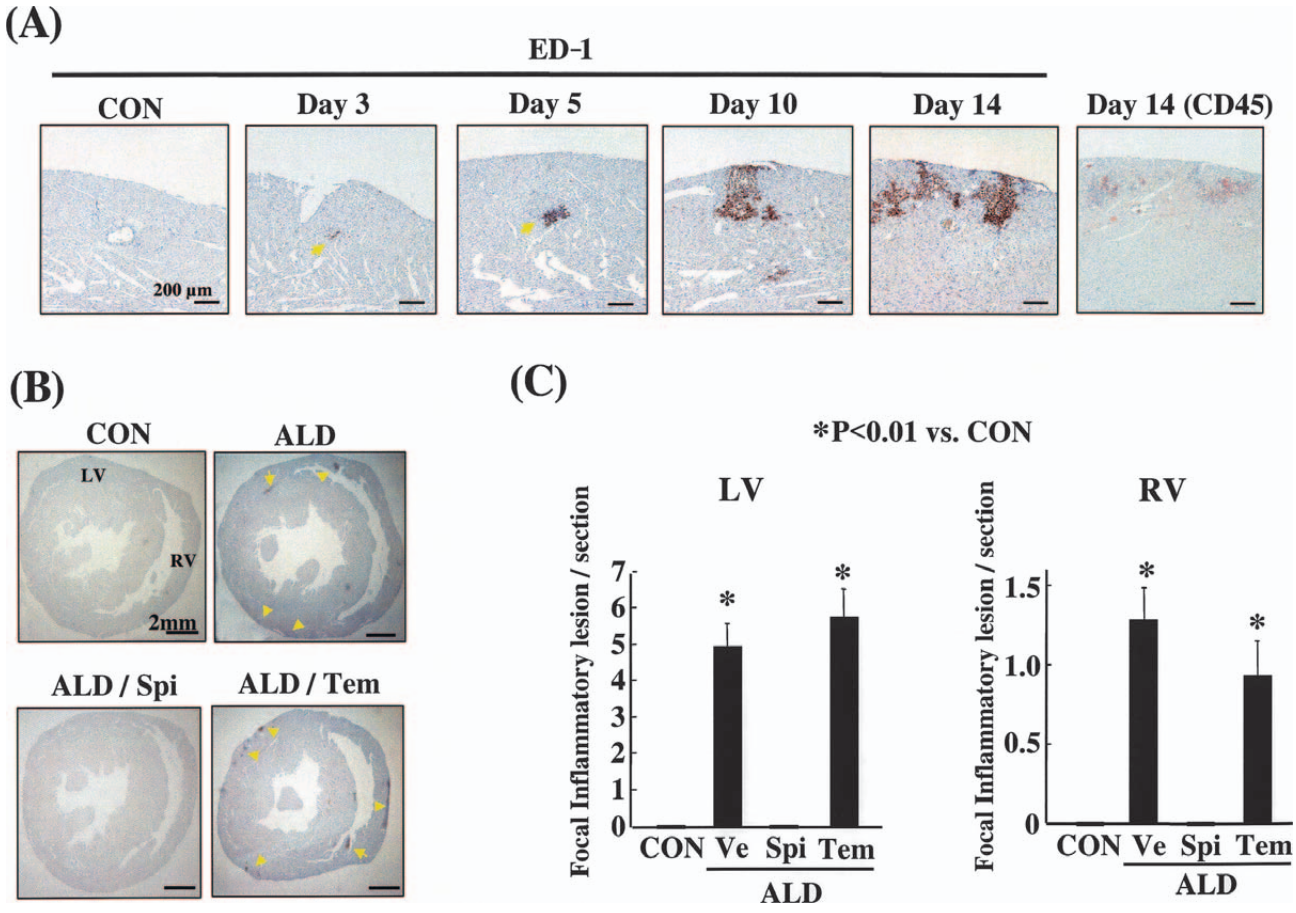

Fig. 3. *Time course of the appearance of focal inflammatory lesions in the heart of ALD-infused rats and the effect of spironolactone and tempol on cardiac focal inflammatory lesions. A: Shows an LV section immunostained with anti-ED-1 antibody at days 3, 5, 10, and 14 after starting ALD infusion. The arrows in sections at days 3 and 5 indicate the appearance of ED-1 positive inflammatory lesions. Day 14 (CD45) indicates an LV section immunostained with anti-CD45 antibodies from a rat infused with ALD for 14 days. Original magnification, ×100. B: Shows representative cardiac sections immunostained with ED-1 antibody from control rats (CON) and rats infused with ALD for 14 days and treated with vehicle (ALD), spironolactone (ALD/Spi) or tempol (ALD/Tem). Original magnification, ×12.5. C: The bar graph shows the number of focal inflammatory clusters per section in the LV or right ventricle (RV). No focal inflammatory lesions were observed in the control group. Focal inflammatory lesions rich in ED-1 positive cells were counted in 4 sections of each LV and RV in individual rats, and the mean value per section was obtained. The abbreviations are the same as in Fig. 2. Each bar represents the mean±SEM (*n* = 6 to 7 per group).*

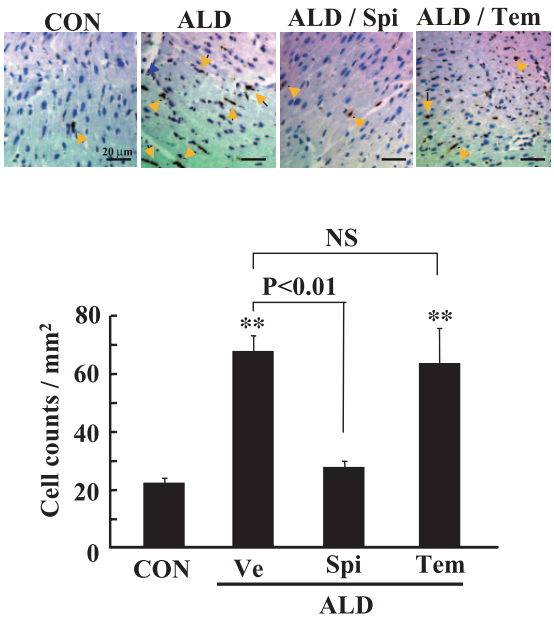

Fig. 4. *Effects of ALD on macrophage infiltration in the interstitium of the LV. The top panel shows representative images of cardiac interstitial macrophage infiltration. Sections were immunostained with anti-ED1-antibody. Arrowheads show ED-1 positive cells in the interstitium of the LV. The abbreviations are the same as in Fig. 2. Each bar represents the mean±SEM (*n = *6 to 7 per group). **p < *0.01* vs. *the control group.*

Technology, Andover, USA) as previously described in detail (*23*). In brief, rats were lightly anesthetized by intraperitoneal administration of ketamine HCl (50 mg/kg) and xylazine HCl (10 mg/kg), and were held in the half left-lateral position. M-mode tracings were recorded through LV anterior and posterior walls (AW and PW, respectively) at the papillary muscle level to measure LV end-diastolic dimension (LVEDD), LV end-systolic dimension (LVESD), fractional shortening (FS), LV ejection fraction (EF), LV AW thickness at end diastole, and PW thickness at end diastole.

Statistical Analysis

All data are presented as the mean±SEM. Statistical significance was determined with one-way ANOVA, followed by Duncan multiple range comparison tests, using Super ANOVA (Abacus Concepts, Inc., Berkeley, USA). Differences were considered statistically significant at a value of $p<0.05$.

Results

Effect of ALD Infusion on Blood Pressure

Figure 1 shows the time course of SBP in ALD (0.75 μg/h)-infused rats. ALD infusion to rats did not increase blood pressure at 1, 3, or 7 days. However, the blood pressure of rats receiving 14 days of ALD infusion was slightly but significantly higher than that of the control group (124±3 *vs.* 112±2 mmHg; $p<0.05$), and this slight increase in blood pressure by 14 days of ALD infusion was completely prevented by treatment with spironolactone (115±1 mmHg) or tempol (111±2 mmHg) ($p<0.05$).

Effects of ALD on Cardiac Hypertrophy

As shown by echocardiographic analysis in Fig. 2A, LV AW and PW thickness (1.51±0.04 mm and 1.56±0.04 mm, respectively) in rats infused with ALD for 14 days were significantly greater than those in CON (1.42±0.02 mm and 1.40±0.03 mm, respectively; $p<0.01$). The increase in AW and PW thickness by ALD was completely suppressed by spironolactone, but not by tempol. Other echocardiographic parameters, including LVEDD, LVESD, FS and EF, were not significantly changed by ALD infusion (Table 1).

Figure 2B shows the results for cardiomyocyte CSA. Cardiomyocyte CSA in ALD-infused rats was greater than that in controls (233.0±7.2 *vs.* 172.8±3.1 μm^2; $p<0.01$), and this increase was suppressed by spironolactone (169.3±3.2 μm^2; $p<0.01$), but not by tempol (220.1±2.2 μm^2).

Formation of Cardiac Focal Inflammatory Lesion by ALD Infusion

As shown in Fig. 3A, ALD infusion caused the appearance of focal inflammatory lesions characterized by ED-1-positive cells (macrophages) at day 3 after the start of infusion, such focal inflammatory lesions were gradually enlarged, and severe focal inflammatory lesions were detected at day 14. These lesions were also positively stained with anti-CD45 (common leukocyte antigen), indicating leukocyte infiltration.

We examined the effect of spironolactone and tempol (1 mmol/l in the drinking water) on cardiac focal inflammatory lesions by ALD. As shown in Fig. 3, spironolactone completely prevented the formation of focal inflammatory lesions by ALD infusion, while tempol failed to prevent it.

We also examined the effect of ALD on cardiac interstitial macrophage infiltration. As shown in Fig. 4, ALD induced a significant increase in cardiac interstitial macrophage infiltration, and this infiltration was completely blocked by spironolactone treatment, but was not affected by tempol at all.

Effect of *N*-Acetylcysteine on ALD-Induced Cardiac Inflammation and mRNA Expression

As described above, administration of tempol (1 mmol/l in the drinking water), a SOD mimetic, despite the reversal of blood pressure elevation by ALD, failed to suppress ALD-induced cardiac hypertrophy and formation of monocyte/macrophage-

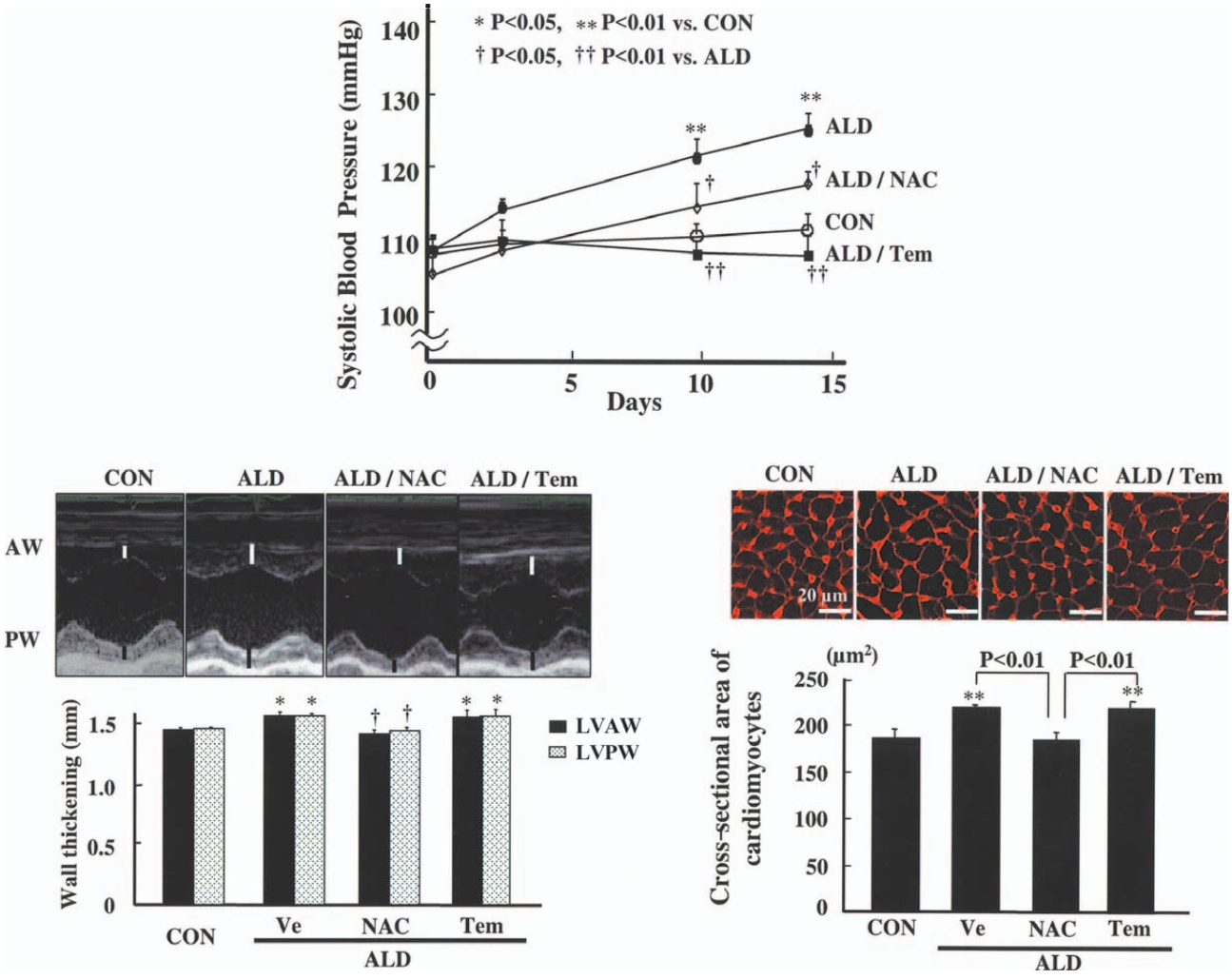

Fig. 5. *Effect of* N-*acetylcystein (NAC) on blood pressure elevation and cardiac hypertrophy by ALD. The upper panel shows the time course of the blood pressure of control rats and ALD-infused rats treated with NAC or tempol. The left lower and right lower panels indicate the LV anterior (AW) and posterior wall (PW) thickening estimated by echocardiography and cardiomyocyte CSA of each group of rats, respectively. CON, control group; ALD, aldosterone-infused group; ALD/NAC, aldosterone-infused group treated with NAC (200 mg/kg/day, i.p.); ALD/Tem, aldosterone-infused group treated with tempol (3 mmol/l in the drinking water). Data are expressed as the mean±SEM (*n = *5 to 7 per group).*

positive inflammatory lesions. To confirm that oxidative stress was indeed not involved in ALD-induced cardiac hypertrophy and inflammatory lesion formation, we examined the effect of NAC, another inhibitor of oxidative stress with a mechanism different from that of tempol, on cardiac injury induced by ALD. Furthermore, we examined the effect of a higher concentration of tempol (3 mmol/l in the drinking water) on cardiac injury induced by ALD. As shown in Fig. 5, NAC treatment significantly suppressed ALD-induced blood pressure elevation ($p < 0.05$), but the suppressive effect by NAC was weaker than that by a high concentration of tempol ($p < 0.01$). However, echocardiographic analysis and measurement of the cardiomyocyte CSA indicated that the cardiac hypertrophy induced by ALD was completely blocked by NAC but not by a high concentration of tempol. Moreover, as shown in Fig. 6, NAC treatment, but not tempol, inhibited the formation of focal inflammatory lesions in the LV and right ventricle (RV) of ALD-infused rats.

We also examined osteopontin, and monocyte chemoattractant protein-1 (MCP-1) mRNA expression in the LV of ALD-infused rats. As shown in Fig. 7A, both osteopontin and MCP-1 mRNA began to increase from several days of ALD infusion. Figure 7B shows that the increases in LV osteopontin and MCP-1 mRNA expression by ALD were significantly prevented by treatment with NAC but not with tempol.

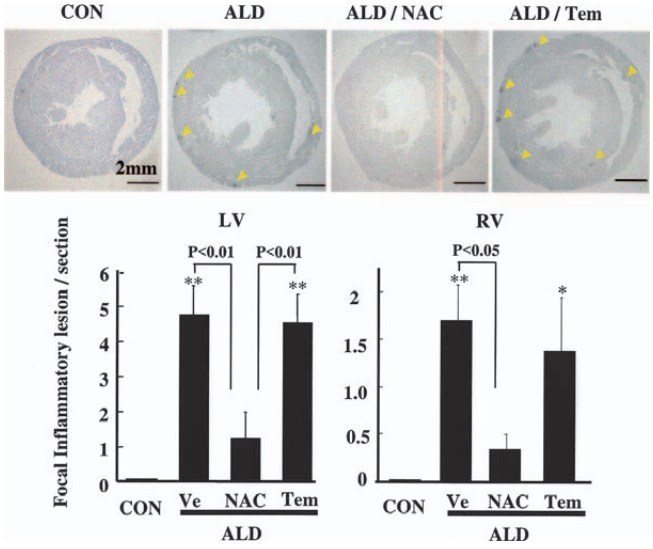

Fig. 6. *Effect of* N*-acetylcystein (NAC) on induction of cardiac focal inflammatory lesions by ALD. The upper panels show representative cardiac sections immunostained with ED-1 antibody from each group of rats. Lower panels indicate the number of LV and RV focal inflammatory lesions immunostained with ED-1 antibody in over 14 days of ALD infusion in rats. CON, control group; ALD, aldosterone-infused group; ALD/NAC, aldosterone-infused group treated with NAC (200 mg/kg/day, i.p.); ALD/Tem, aldosterone-infused group treated with tempol (3 mmol/l in the drinking water). Data are expressed as the mean±SEM (*n = *5 to 7 per group).*

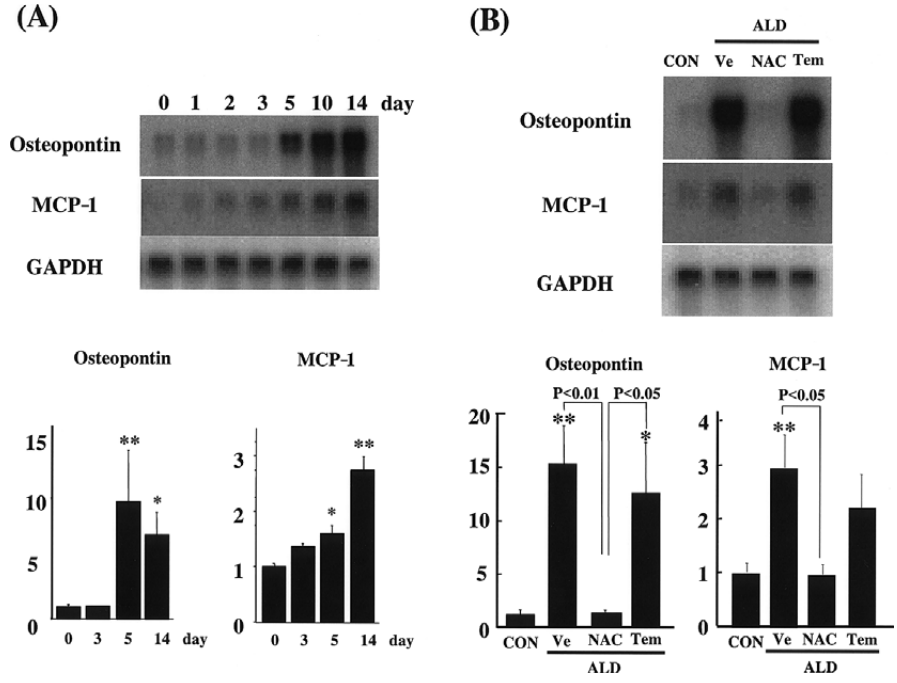

Fig. 7. *Time course of LV osteopontin and MCP-1 mRNA levels during ALD infusion (A) and effect of NAC and tempol on their mRNA expressions (B). A: The upper panel shows representative autoradiograms of osteopontin, MCP-1 and GAPDH mRNA from the LV of rats infused with ALD for various numbers of days. The lower bar graphs indicate quantitative data on LV osteopontin and MCP-1 mRNA at 3 or 5 days of ALD infusion.* *p < *0.05,* **p < *0.01* vs. *0 days. B: The upper and lower panels show representative autoradiograms and quantitative data on LV osteopontin and MCP-1 mRNA from controls and rats infused with ALD for 14 days and treated with NAC or tempol. The abbreviations are the same as in Fig. 6. Data are expressed as the mean±SEM (*n = *5 to 7 per group).* *p < *0.05,* **p < *0.01* vs. *CON.*

Discussion

The present study was undertaken to elucidate the short-term effect of excess ALD alone on blood pressure, cardiac hypertrophy and inflammation of normal rats. It is noteworthy that ALD, while causing a slight elevation of blood pressure, also caused a significant increase in cardiac hypertrophy and induced severe cardiac inflammatory lesions. The ALD-induced cardiac injury was inhibited by NAC but not by tempol. These results show that excess ALD, independently of blood pressure, plays a pivotal role in cardiac remodeling *via* oxidative stress.

Beginning in the 1930s, numerous studies have documented that mineralcorticoid with the addition of excess dietary salt and the removal of one kidney could remarkably elevate blood pressure and induce cardiac damage (*24*). Accumulating *in vivo* evidence has shown that ALD infusion induces severe blood pressure elevation, cardiac hypertrophy, inflammatory responses, and fibrosis (*8*, *9*, *17*, *25–29*). However, previous studies on ALD infusion were mostly performed in rats with excess sodium loading, uninephrectomy, or both (*8*, *9*, *17*, *25–29*). Therefore, the previous studies have not clarified whether cardiac injuries by ALD were mediated by excess ALD itself, or by sodium retention or severe hypertension. The effect of ALD infusion alone on cardiac injury, particularly the short-term effect of ALD infusion, is poorly understood. This fact encouraged us to examine the effect of ALD infusion on cardiac injury in normal rats not subjected to sodium loading or uninephrectomy.

In the present study, we found that ALD infusion alone very slowly and only slightly increased the blood pressure of normal rats. Our data differed from the previous findings (*8*, *9*, *17*, *25–28*) on the severe elevation of blood pressure by ALD infusion in rats with salt loading, uninephrectomy, or both. The difference between our present observations and previous findings shows that excess sodium plays a pivotal role in severe hypertension induced by excess ALD, as previously reported. However, in the present study, although 14-day ALD infusion induced a minor elevation of blood pressure, it also significantly induced a significant increase in cardiac hypertrophy, as shown by the increase in LV wall thickness and cardiomyocyte area. Thus excess ALD had a potent effect on cardiac hypertrophy, but only a slightly one on blood pressure.

Previous reports have indicated that ALD infusion in rats that underwent salt loading, uninephrectomy or both produced cardiac inflammatory lesions mainly consisting of macrophages/monocytes (*8*, *9*, *27*, *29*, *30*). However, it cannot be ruled out that the severe cardiac inflammatory responses induced by ALD infusion in these studies may have been due to salt loading or severe hypertension. In the present study, we found that excess ALD, without saline intake or uninephrectomy, potently caused cardiac focal inflammatory lesions at as early as 3 days after the infusion, followed by a very small blood pressure elevation. This onset of focal macrophage/monocyte infiltration into the cardiac tissue by ALD infusion was associated with the rapid induction of cardiac MCP-1 and osteopontin gene expression, and was followed by tissue fibrosis. Thus, our results provide evidence that excess ALD alone directly induces cardiac inflammation under a normal salt diet.

A previous study indicated that cardiac inflammation by ALD infusion with salt loading and uninephrectomy was mediated by oxidative stress, as shown by the inhibition of ALD-induced cardiac inflammatory responses by NAC or pyrrolidine dithiocarbamate (PDTC) (*9*). However, no information is available on the possible implication of oxidative stress in cardiac injury by ALD infusion alone. In the present study, therefore, we examined the contribution of oxidative stress to ALD-induced cardiac hypertrophy and inflammation. For this purpose, in the first experiment, we used tempol, a superoxide dismutase mimetic, to examine the possible contribution of superoxide to ALD-induced blood pressure elevation and cardiac damage. Although tempol completely blocked the rise in blood pressure by ALD infusion, it failed to inhibit ALD-induced cardiac hypertrophy, cardiac inflammatory lesions, or MCP-1 and osteopontin gene expressions. These observations indicated that superoxide play a major role in the slight elevation of blood pressure by ALD, but only a minor role in the cardiac hypertrophy and inflammatory responses induced by ALD. To further elucidate the role of oxidative stress in ALD-induced cardiac damage, in the second experiment, we used another antioxidant, NAC. Endogenous thiols (sulfhydryl-containing compounds), such as glutathione, serve as antioxidants in cells. NAC is a thiol, a mucolytic agent, and a precursor of L-cysteine and reduced glutathione. NAC is a source of sulfhydryl groups in cells and a scavenger of free radicals such as OH• and H_2O_2 but not superoxide, as noted in a previous review (*31*). Thus, NAC and tempol exhibit different actions on reactive oxygen species. Of note, in contrast to tempol, NAC significantly prevented ALD-induced cardiac hypertrophy and inflammation, and cardiac MCP-1 and osteopontin gene expression. Therefore, it seems that ALD-induced cardiac hypertrophy and inflammatory lesions are mediated by oxidative stress that is inhibited by NAC but not by tempol. However, further studies are needed to elucidate the detailed role of oxidative stress in ALD-induced cardiac injury.

In summary, our present work provide evidence that excess ALD alone, without salt loading and uninephrectomy, potently induces cardiac hypertrophy and inflammatory lesions despite minor blood pressure elevation. These effects by excess ALD alone were mediated by oxidative stress that was inhibited by NAC but not by tempol. In conclusion, our present work provides insight into the pathophysiological role of ALD in cardiac diseases, as well as a rationale for the usefulness of combination therapy with an ALD blocker and renin-angiotensin blocker in cardiac diseases. However, fur-

ther studies will be needed to elucidate the role of ALD in cardiac hypertrophy and remodeling.

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