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Macrophage depletion protects against endothelial dysfunction and cardiac remodeling in angiotensin II hypertensive mice

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ABSTRACT

Objective: Hypertension is associated with a low-grade systemic inflammation in cardiovascular system. Macrophage infiltration may initiate an inflammatory process that contributes to vascular and ventricular remodeling in hypertensive human and mice. The present study investigated the effect of chemical depletion of macrophage using liposome encapsulated clodronate (LEC) on cardiac hypertrophy and remodeling in angiotensin (Ang) II hypertensive mice.

Methods: C57BL/6 mice received an Ang II (1.1 mg/kg/day with a minipump) infusion for 2 weeks to induce hypertension. Endothelium-dependent relaxation (ED) was examined by organ bath, hematoxylin and staining and Masson-Trichrome staining were used to evaluate aorta and cardiac hypertrophy and fibrosis.

Results: Ang II infusion significantly increased systolic blood pressure (SBP), cardiac hypertrophy and fibrosis, and impaired EDR accompanied by increased macrophage infiltration in the heart. Treatment with LEC significantly lowered Ang II-induced cardiac hypertrophy and fibrosis and cardiac macrophage infiltration, and improved EDR with a mild reduction in SBP. Ang II increased the expression of inflammatory cytokines tumor necross factor alpha and interleukin 1 beta and profibrotic factors transforming growth factor beta 1 and fibronectin in the heart, with was reduced by LEC treatment. Treatment with LEC prevented Ang II-induced the phosphorphorylation of ERK1/2 and c-Jun-N-terminal kinase.

Conclusions: Our study suggests that cardiac macrophage may be critical for hypertensive cardiac hypertrophy and remodeling, the underlying mechanisms may involve initial heart inflammation and the activation of hypertrophic MAPKs pathway.

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KEYWORDS
Angiotensin II; cardiac hypertrophy and remodeling; hypertension; macrophage; mapk

Introduction

Hypertension is a major risk factor for cardiovascular disease. Hypertension leads to structure changes in the heart and vasculature through the process of cardiac and vascular remodeling, including cardiac hypertrophy, fibrosis, and vascular wall thickness. Cardiac hypertrophy, particularly left ventricular hypertrophy, is a hallmark of hypertensive heart disease, and one of the most robust and validated prognostic markers in hypertension (1).

Hypertension is associated with a chronic low-grade inflammatory process in the vasculature and its target organs (2,3). Hypertensive cardiac hypertrophy may result from the mechanical force due to high blood pressure as well as inflammation caused by immune cell infiltration (4). Immune cells, such as T lymphocytes, monocytes/macrophages, have been found in the vascular and cardiac tissues in a variety of experimental hypertensive animal models (5,6). Macrophages produce/release inflammatory cytokines, such as tumor necrosis factor (TNFα) and interleukin (IL) 1β, which may induce vascular and myocardial inflammation, fibrosis, oxidative stress, and extracellular matrix proteins in the vasculature and heart, thus promoting vascular and cardiac remodeling (6,7). Although macrophages are implicated in many features of human health and diseases, their roles in the pathogenesis of hypertensive heart diseases are complex and controversial. The depletion of macrophages can be both protective and detrimental for hypertensive heart disease (5,8,9).

Inappropriate activation of the renin angiotensin (Ang) system is implicated in the pathogenesis of hypertension and hypertensive end-organ damage (10,11). Ang II is a major mediator to induce cardiac and vascular remodeling and inflammation (11). Besides being a potent vasoactive peptide, Ang II also increases the recruitment of monocytes/macrophages and inflammation in its target organs, such as the heart, kidney, and vascular system (12). Liposome-encapsulated clodronate (LEC) is a chemical compound which is widely used for the depletion of macrophages from

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various organs and tissues *in vivo* (13–15). In the present study, we investigated the effect of macrophage depletion on endothelial function and cardiac hypertrophy and remodeling in Ang II hypertensive mice.

Materials and methods

Animal studies

Six-week-old C57BL/6 male mice were purchased from Beijing Charles River Animal Laboratory (Beijing, China). The mice were housed in the animal care facility with a light-dark cycle of 12:12 hours and had free access to standard mouse chow and tap water. All animal protocols complied with the international standard guidelines for the care and use of laboratory animals and were approved by the Institutional Animal Care and Use Committee of Shenyang Medical University. Before the experiments were conducted, the mice were allowed to adapt to new environment for 2 weeks. The mice were divided into four groups and treated for 4 weeks as follows: 1) normal control group (Ctr): the mice were implanted with an empty osmotic mini-pump (Alzed model 1002D, DURECT Inco., Cupertino, CA) plus PBS-liposome treatment (Liposoma B.V., Amsterdam, Netherlands, n = 7); 2) normal mice with LEC treatment (LEC): the implantation with an empty osmotic mini-pump plus LEC treatment (Liposoma B.V., Amsterdam, Netherlands, n = 7); 3) Ang II hypertensive mice (Ang II): the implantation with an osmotic mini-pump of Ang II with PBS-liposome treatment (n = 7) (4); Ang II hypertensive mice with LEC treatment (Ang II/LEC): the implantation with an osmotic mini-pump of Ang II plus LEC treatment (n = 7). Cardiac hypertrophy was induced by the infusion of pressor dose of Ang II (1.4 mg/kg/day, Sigma Aldrich Inco., St. Louis) for 2 weeks. Briefly, the mice were anesthetized by sodium pentobarbital (50 mg/kg I.P). An incision was made in the midescapular region, and an osmotic mini-pump was implanted. It has been shown that the infusion of Ang II at this pressor dose into mice for 1–2 weeks can significantly increase blood pressure and induce cardiac hypertrophy and vascular injury (16). Macrophage depletion was done through the tail vein injection of LEC at 200 μl/kg body weight. LEC was injected on the day before the implantation of an osmotic mini-pump, and the injections were repeated every 3 days until the end of the experiment. The mice in control groups (with or without Ang II infusion) were injected with an equal volume of PBS-liposome at a similar interval. On the second day of LEC injection, a drop of blood was taken from the tail vessels for blood smear and stained with Giemsa (GS500; Sigma Aldrich, St. Louis). The monocytes, granulocytes and lymphocytes were identified according to their nuclear morphology, and a total of 350 white blood cells per smear were counted. Systolic blood pressure (SBP) was measured by the tail-cuff method (Softron Biotech Inco. Beijing) in the conscious mice. The mice were placed in a quiet and dark room. SBP were measured on the day before the mini-pump implantation and the first and second weeks after Ang II infusion. Before blood pressure measurement, the mice were trained daily for five consecutive days to adapt to blood pressure measurement. At least five successive readings of blood pressure for each mouse were taken and averaged as one measurement. At the end of the experiment, the mice were euthanized by overdose anesthetized agents (sodium pentobarbital 100 mg/kg I.P), the heart was isolated and weighted. The ratio of heart weight (HW)/body weight (BW) was calculated and used as an index of cardiac hypertrophy.

Histological analysis

A piece of left ventricle was fixed in 4% paraformaldehyde in PBS buffer and embedded in paraffin. The samples were cut into 4-μm thick section and stained with hematoxylin and eosin (Sigma Aldrich Inco., St. Louis). To evaluate left ventricular hypertrophy, at least four images from four randomly selected fields per slide were taken, and the cross-sectional area of 100 cardiomyocytes per field was measured using quantitative digital image analysis system (Media Cybernetics, Rockville). In addition, Masson-trichrome (Sigma Aldrich Inco.) was performed to assess cardiac fibrosis. Cardiac collagen contents were measured in eight randomly selected fields in two nonconsecutive slides per sample, using Image Pro Plus image analysis system. The data was expressed as the percentage of positive collagen areas (blue) with total selected areas. All histologic samples were blind to the reviewers who were not aware of the groups to which the mice belonged.

Immunohistochemistry analysis

The paraffin-embedded heart tissues were prepared and cut into a 4-μm thick section as described above. After the deparaffinization, the sections were incubated with retrieval solution at 60°C for 30 minutes for antigen retrieval, then the sections were incubated with blocking solution of 5% serum for 1 h at room temperature. The sections were incubated with the primary antibody against F4/80 (1:100 dilution with TBST buffer, Abcam Inco.) overnight at 4°C, followed by the incubation with secondary antibody was biotinylated horse anti-mouse IgG (Vector Laboratories). A Vectastain Elite ABC Kit (Vector Laboratories) was used according to the manufacturer's protocol. The nuclei were stained with DAPI. F4/80 positive cells (monocyte/macrophage) in the heart were viewed and counted by an experienced reviewer who was blind to experimental groups by using a fluorescence microscope. At least five images per slide were examined and averaged as a single value, the number of positive F4/80 staining cells was expressed by per mm^2 area of selected view area.

Determination of endothelium-dependent relaxation (EDR)

EDR to acetylcholine in the aortic rings was determined by an organ chamber bath (DMT Inco., Denmark), as previously described (17). Briefly, the aorta was removed and cleaned of

adherent tissues. The descending throracic aorta was cut into a 3 mm ring. After equilibrated for 1 h in 1 g of resting tension, the aortic rings were contracted twice by 60 mmol/L KCl solution with 30-min interval. The rings were precontracted with norepinephrine (about 30 nmol/L), which induced about 70% of maximal contraction, and then cumlative doses (10^{-9} to 10^{-5} mol/L) of acetylcholine were added into the organ bath, and acetylcholine-induced vasorelaxation was recorded. Relaxation to acetylcholine was expressed as a percentage inhibition of norepinephrine-induced vasoconstriction. The maximal relaxation to acetylcholine (Emax) and the acetylcholine concentration required for induction of a half-maximal response (ED_{50}) were determined from the concentration–response curve, using best fit to a logistic sigmoid function.

Western blot

A piece of left-ventricle tissue was homogenized with lysis buffer containing 1 mM PMSF, 10 μg/ml aprotinin and 10 μg/ml leupeptin. After the homogenization, an aliquot of supernatant was used for the protein measurement with Bio-Rad protein assay. Thirty μg of proteins was separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with the primary antibodies against c-Jun N-terminal kinase (JNK, Cell signaling), phosphor-JNK, p38 mitogen-activated protein kinase (MAPK), phosphor-p38 MAKP, p42/p44 MAPK and phosphor-p42/44 MAPK (Cell Signaling Inco.), transforming growth factor (TGF) β1, fibronectin, TNFα, IL1β (Santa Cruz Biotech Inco.) at 4°C overnight. The membranes were incubated with horseradish peroxidase conjugated secondary antibody for 1 h at room temperature. The signal was detected by enhanced chemiluminescence (ECL) using hyperfilm and ECL reagent (Santa Cruz Biotech Inco.). The membranes were reblotted for β-actin (Santa Cruz Biotech Inco.) to serve as a loading control. The membranes for the determination of phosphor-JNK, phosphor-ERK1/2 and phosphor-p38 MAPK were reblotted with their corresponding non-phosphor forms to serve as a loading control. The data was normalized to β-actin or corresponding control protein and expressed as a fold increase versus control group.

Statistical analysis

The results were expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed by ANOVA with Bonferonni's correction for multiple comparisons. Significance was assumed at $p < .05$.

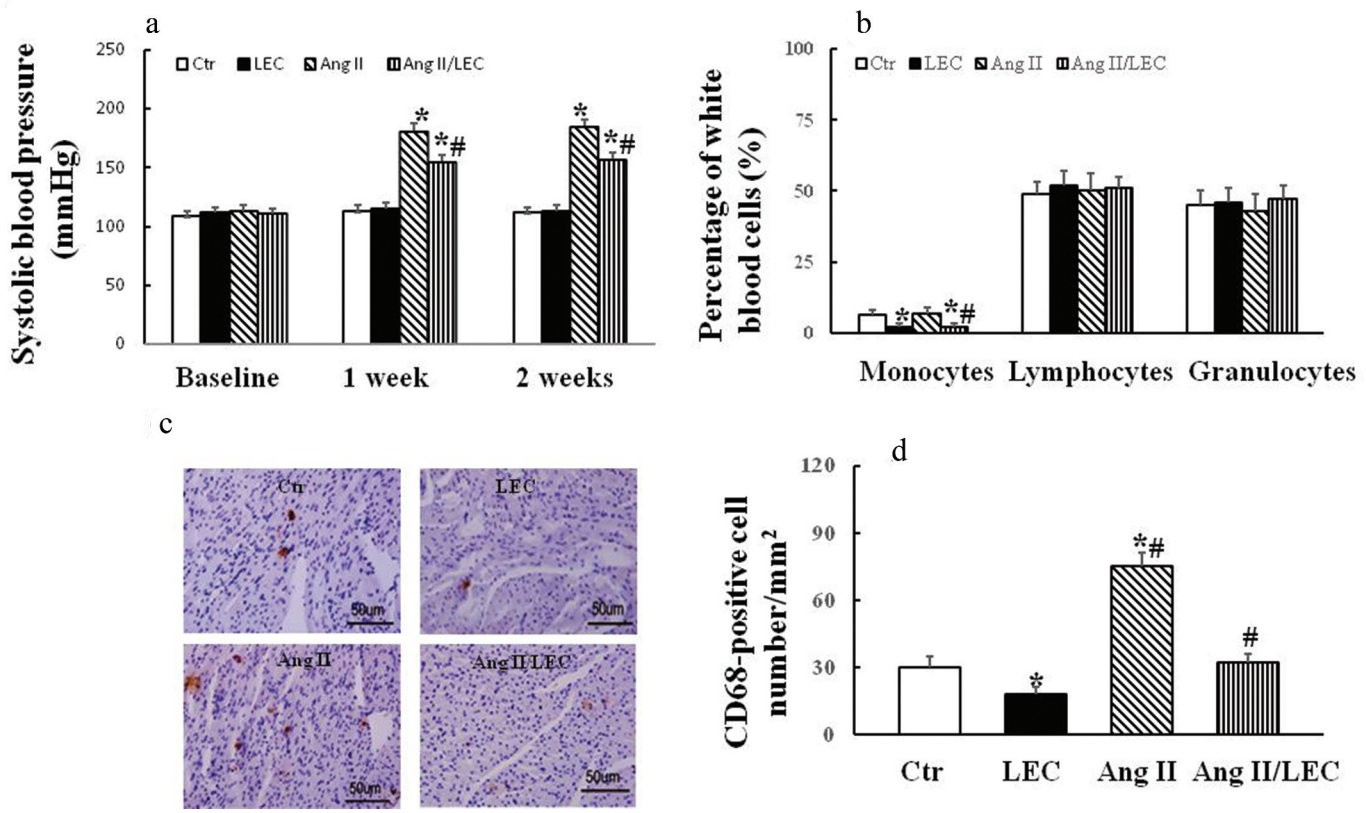

Figure 1. Liposome-encapsulated clodronate (LEC) lowered systolic blood pressure (A), circulating monocytes (B) and cardiac macrophages (C&D) in Ang II infusion mice. The representative images of cardiac CD68 expression assessed by immunohistochemistry (C); Quantitation of cardiac CD68 expression (D). Ctr: normal mice treated with PBS-liposome, LEC: normal mice treated with LEC, Ang II: the mice treated PBS-liposome plus Ang II for 2 weeks. Ang II/LEC: the mice treated with LEC plus Ang II infusion for 2 weeks. All data was expressed as mean ± SEM. N = 7, *p < .05, vs. ctr group, #p < .05, vs. Ang II group.

Results

LEC reduced SBP and macrophage infiltration in the heart of Ang II hypertensive mice

The infusion of Ang II significantly increased SBP at the first week and maintained high blood pressure for 2 weeks (185 ± 6 vs. 112 ± 4 mmHg in control group, $p < .05$) in the mice. Tretment with LEC mildly but signficantly attenuated Ang II–induced elevation of SBP (157 ± 6 vs. 185 ± 5 mmHg in Ang II mice, $p < .05$, Figure 1A). LEC is an effective drug at depleting circulatory monocytes and tissue macrophages by inducing monocyte/macrophage apoptosis (13). In the present study, the mice were administrated with LEC at a similar way with the dose and interval as reported (8,13). Giemsa-staining showed that treatment with LEC resulted in 60–70% reduction in the circulating monocytes in both control and Ang II mice, but did not significantly affect the circulating lymphocytes and granulocytes (Figure 1B). F4/80 is a unique marker of murine macrophage, as shown in Figure 1C&D, and the number of F4/80 positive cells was significantly increased in the heart of Ang II mice, LEC reduced F4/80 positive cells in both control and Ang II mice. The results suggest that LEC can effectively and specifically deplete the circulating monocytes and tissue macrophages.

Depletion of macrophages by LEC attenuated Ang II–induced cardiac hypertrophy and fibrosis. Ang II infusion for 2 weeks significantly increased heart weight in the mice (495 ± 27 vs. 371 ± 19 mg/100 g body weight in control mice, $p < .05$). LEC did not affect heart weight in control mice but significantly reduced heart weight in Ang II hypertensive mice (426 ± 23 vs. 495 ± 27 mg/100 g body weight; $p < .05$, Figure 2 A&B). HE staining showed that Ang II increased cardiomyocyte sectional area, which was reduced in Ang II/LEC mice (Figure 2 C&D). In addition, Masson's-trichrome staining showed that Ang II significantly increased positive collagen-staining area in the heart tissue, which was partially reduced by LEC treatment (Figure 3A&B). TGFβ1 and its downstream molecule fibronectin are important fibrotic factors to promote cardiovascular remodeling and fibrosis. As shown in Figure 3 C&D, Ang II significantly increased the protein expressions of TGFβ1 and fibronectin in the heart, which was reversed by LEC treatment.

Depletion of macrophages by LEC improved endothelial function and reduced proinflammatory cytokine expression in Ang II hypertensive mice. Endothelium-dependent relaxation to acetylcholine was significantly impaired in the aorta of Ang II mice (Emax: 63 ± 4% vs. 97 ± 3% in control mice, $p < .05$; ED50 7.3 ± 0.2 vs. 7.5 ± 0.1 – log M in control mice, $p > .05$). LEC significantly improved acetylcholine-induced endothelium-dependent relaxation (Emax: 81 ± 4 vs. 63 ± 4% in Ang II mice, $p < .05$, ED50 7.5 ± 0.2 vs. 7.3 ± 0.2 -log M in Ang II mice, $p > .05$, Figure 4). Infiltrating macrophages can release inflammatory cytokines and induce inflammation, as shown in Figure 5, and the protein expressions of

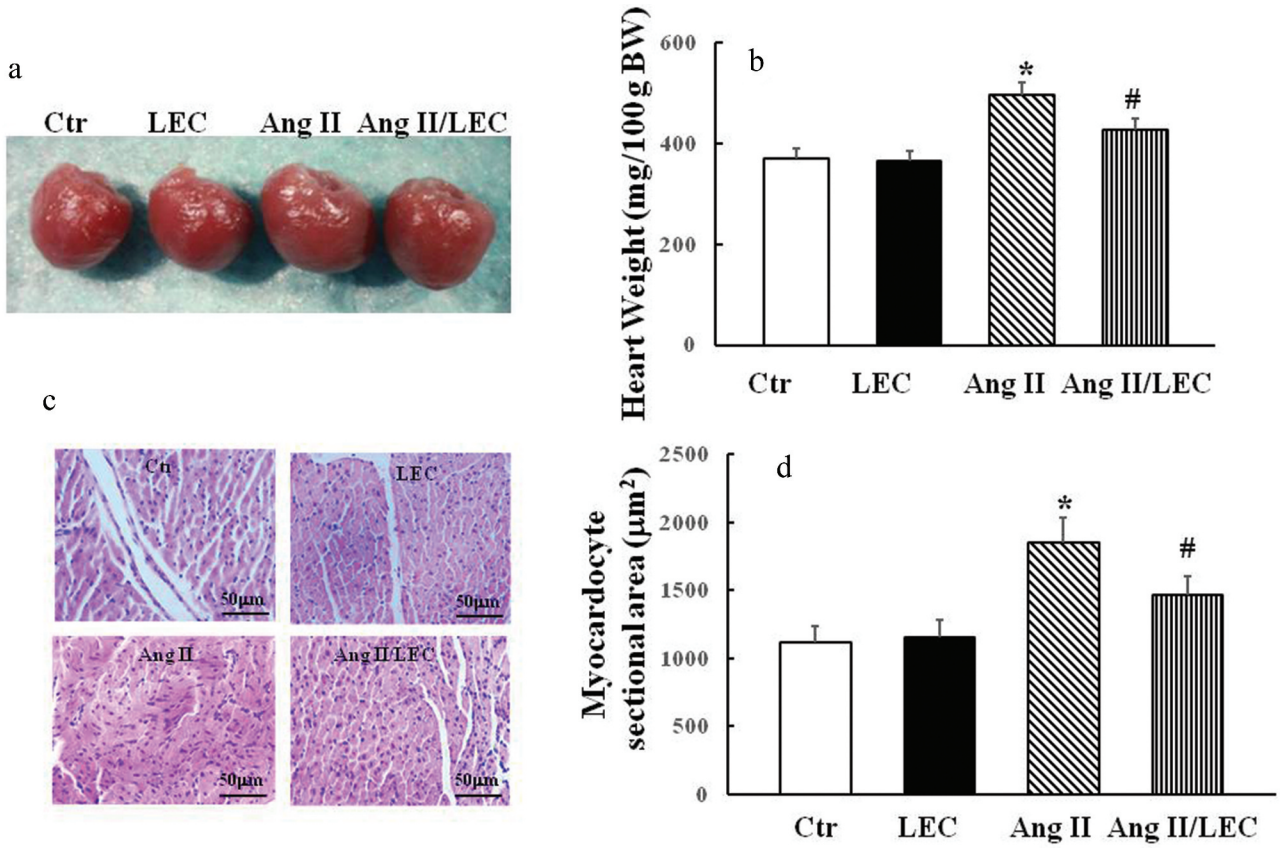

Figure 2. Macrophage depletion by LEC lowered cardiac hypertrophy in Ang II hypertensive mice. Cardiac hypertrophy was assessed by heart weight (A&B) and cardiac myocyte sectional area (C&D). The representative images of cardiomyocyte sectional area assessed by hematoxylin and eosin staining (C). Quantitative analysis of cardiomyocyte sectional area (D). N = 7, *p < .05, vs. Ctr group, #p < .05, vs. Ang II group.

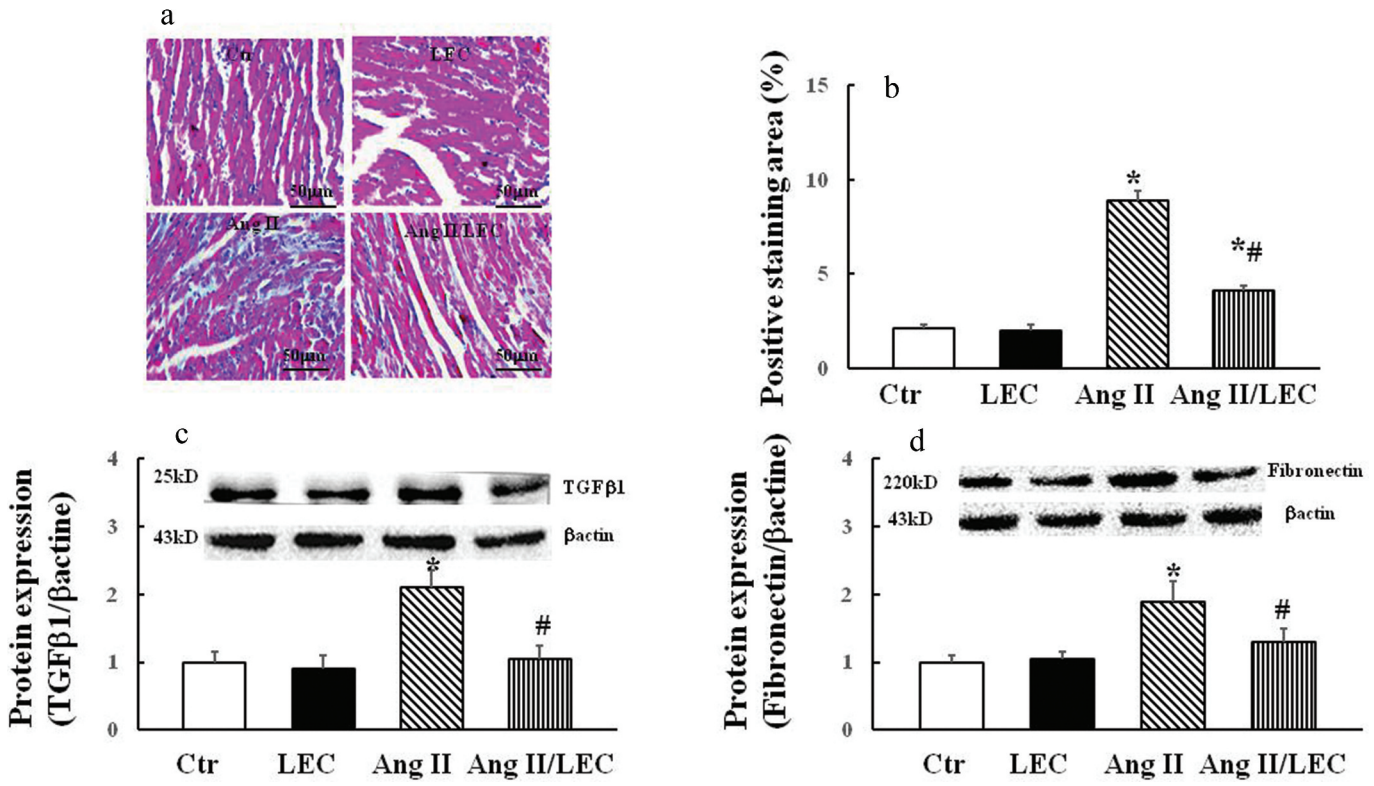

Figure 3. The depletion of macrophages by LEC reduced cardiac fibrosis (A&B) and the expressions of TGFβ (C) and fibronectin (D) in Ang II hypertensive mice. The representative images of cardiac fibrosis assessed by Masson-trichrome staining (A), the quantitative assessment of positive collage-staining area in the heart (B). N = 6–7, *p < .05, vs. ctr group, #p < .05, vs. Ang II group.

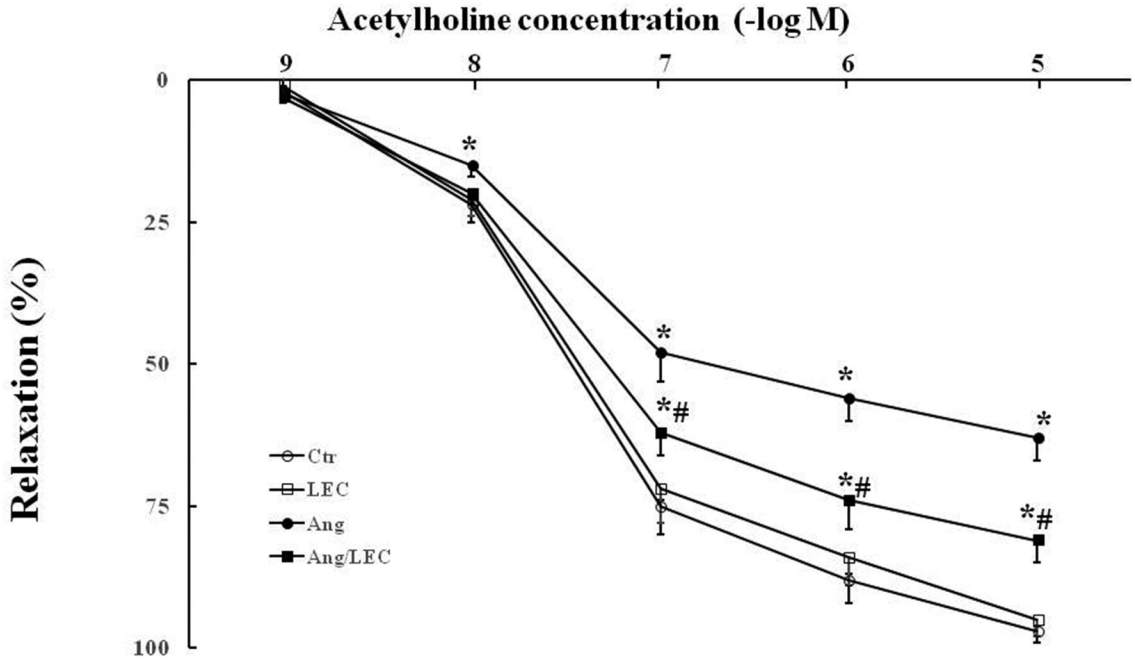

Figure 4. LEC improved endothelium-dependent relaxation to acetylcholine in Ang II hypertensive mice. N = 7, *p < .05, vs. ctr group, #p < .05, vs. Ang II group.

proinflammatory cytokines TNFα and IL1β were significantly increased in the heart of Ang II mice. In addition, treatment with LEC prevented Ang II–induced expressions of these pro-inflammatory cytokines.

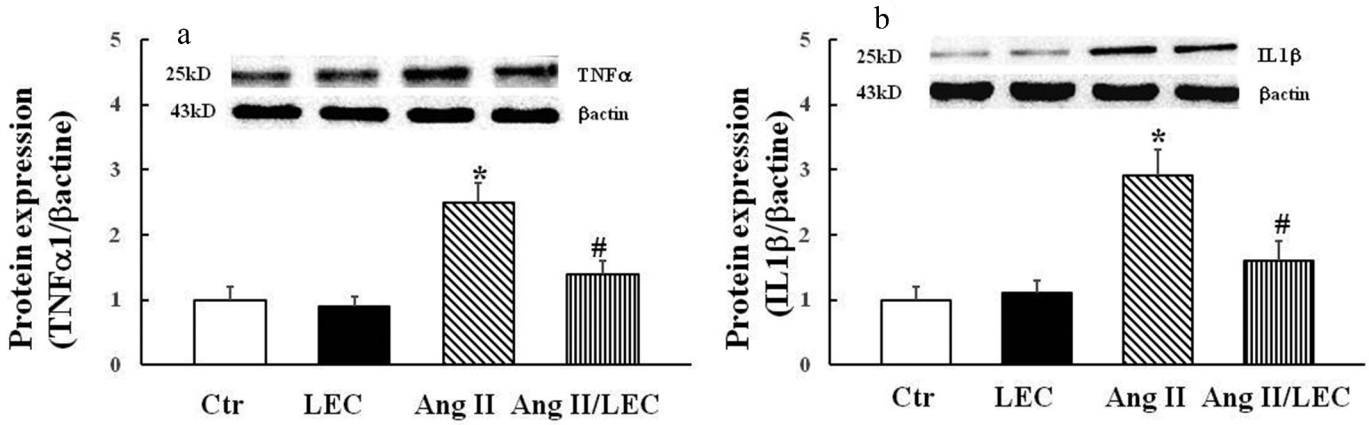

Figure 5. LEC lowered the expression of proinflammatory cytokines TNFα (A) and IL1β (B) in Ang II hypertensive mice. N = 6, *p < .05, vs. ctr group, #p < .05, vs. Ang II group.

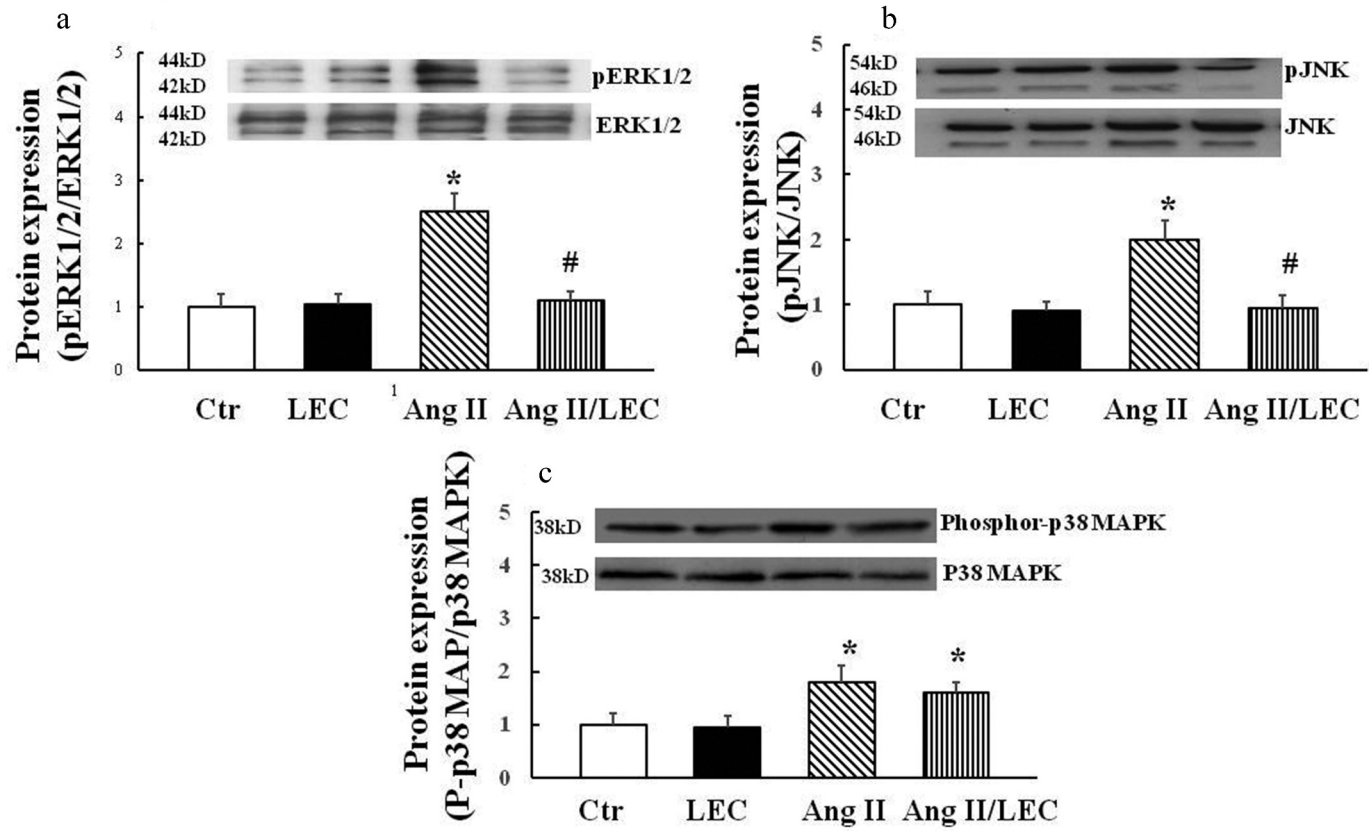

Figure 6. Effects of LEC treatment on the expressions of phosphor-ERK1/2 (A), phosphor-JNK (B) and phosphor-p38 MAP kinase (C) in Ang II hypertensive mice. N = 6, *p < .05, vs. ctr group, #p < .05, vs. Ang II group.

Depletion of macrophages by LEC attenuated Ang II activation of MAPK1/2 and JNK. MAPKs, including ERK1/2, JNK and p38MAPK, have shown to participate in the regulation of cardiac hypertrophy and remodeling (18). We determined the protein expressions of phosphor-ERK1/2, phosphor-p38MAK and phosphor-JNK in the heart. As shown in Figure 6, Ang II significantly increased the protein expressions of phosphor-ERK1/2, Phosphor-p38MAPK, and phosphor-JNK without significant changes in the expressions of total ERK1/2, p38MAPK, and JNK. LEC significantly attenuated Ang II–induced expressions of ERK1/2 and JNK1/2 phosphorylation but did not affect phosphor-p38MAKP expression.

Discussion

Macrophage recruitment is closely associated with hypertensive cardiac remodeling and vascular dysfunction (5,19). In the present study, we demonstrate that chemical depletion of macrophages by LEC inhibits Ang II–induced hypertensive

cardiac hypertrophy and fibrosis and improves endothelial function associated with a mild reduction in blood pressure. These results provide direct evidence that macrophages play a vital role in hypertensive cardiac remodeling.

Hypertension is often associated with structural changes in the heart and vascular system, particularly left ventricular hypertrophy and remodeling (2,16). Besides hemodynamics, nonhemodynamic factors, such as neurohumoral, chronic cardiac inflammation, may also induce cardiac hypertrophy and remodeling in hypertension (6,20). Monocytes/macrophages are major components of the innate immune system, it has been shown that monocytes/macrophages are recruited in the heart and vessel wall in various hypertensive animal models (21–23). The proinflammatory cytokines released by macrophages, such as TNFα, can promote vascular dysfunction and cardiac hypertrophy and remodeling (9,24). TNFα deficiency attenuates cardiac hypertrophy and fibrosis in Ang II hypertensive mice (25). Recently, we have shown that TNFα knockout reduces cardiac hypertrophy and improves vascular function in DOCA-salt hypertensive mice (26). The present study shows that the depletion of cardiac macrophages with LEC attenuates Ang II–induced cardiac hypertrophy and the expressions of proinflammatory cytokines TNFα and IL1β. Thus, these results suggest that macrophages release the proinflammatory cytokines, such as TNFα, which may importantly contribute to hypertensive cardiac hypertrophy and remodeling.

MAPKs are cytosolic-signaling proteins, which can modulate various cellular processes, such as cell growth and cell size regulation, in response to wide extracellular stimuli (18). It has been shown that MAPKs, particularly ERK1/2, play a critical role in cardiovascular remodeling in various cardiovascular diseases (18,27,28). Inflammation, oxidative stress and Ang II have been shown to stimulate the phosphorylation (activation) of MAP kinases, including ERK1/2, JNK, and p38 MAPK (18,27). The present study shows that Ang II increases the phosphorylation of three types of MAP kinases, including ERK1/2, JNK, and p38 MAP kinase in the heart. In addition, the depletion of macrophages by LEC attenuates Ang II–induced phosphorylation of ERK1/2 and JNK associated with a reduction in Ang II–induced cardiac hypertrophy and remodeling. As the macrophage-derived inflammatory cytokines, such as TNFα, MCP1, and ICAM1, have been shown to activate MAPKs (25), we therefore surmise that macrophages release inflammatory cytokines, which may activate MAPK pathways to induce cardiac hypertrophy and remodeling in Ang II hypertension.

Ang II–induced cardiac remodeling is linked to myocardial fibrosis (29). Ang II can directly induce cardiac fibrosis via stimulating the expressions of profibrotic factors and extracellular matrix proteins (30). In addition, Ang II can also promote cardiac fibrotic process via the induction of cardiac inflammation and hemodynamic stress (31). It has been shown that proinflammatory cytokine TNFα can stimulate fibrotic signaling TGFb1/SMAD2/3 signaling. The present study shows that the depletion of macrophage by LEC significantly inhibits Ang II–induced cardiac fibrosis and reduces the expressions of fibrotic factors TGFβ1 and fibronectin.

Limitation: The present study has several limitations. Firstly, treatment with LEC not only attenuates cardiac hypertrophy and remodeling but also slightly lowered blood pressure. As hemodynamics per se is an important factor to promote cardiac hypertrophy and remodeling (32), the present study does not design a control group of blood pressure. Thus, it cannot be excluded that LEC attenuation of cardiac hypertrophy and remodeling is secondary to its effects on blood pressure. It has been reported that antihypertensive agent hydralazine normalizes blood pressure but only slightly attenuates cardiac hypertrophy in Ang II hypertension (33). In the present study, LEC markedly reduced cardiac hypertrophy (60%), but only mildly reduced blood pressure. Thus, we think that LEC inhibition of cardiac remodeling may be due to both the improvement of hemodynamics and a direct effect on the depletion of macrophage. Secondly, blood pressure measurement using tail cuff method may cause the animal stress, which may affect the accuracy of blood pressure. Using well-trained animals before the experiment, like the present study, may increase the data reliability. Thirdly, LEC is a chemical that depletes macrophages in the whole body rather than specificly on the heart. The depletion of macrophages in other organs may affect cardiac remodeling via changes in blood pressure or neuroendocrine system.

In summary, the present study provides direct evidence showing that cardiac macrophages play an important role in cardiac hypertrophy and remodeling in Ang II hypertensive mice. Chemical depletion of cardiac macrophage can reduce cardiac inflammation and protect against hypertensive cardiac injury and remodeling. Our results suggest that cardiac macrophages may be a new target for the prevention and treatment of hypertensive heart diseases.

Acknowledgments

Not applicable

Disclosure Statement

All authors declare no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

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Data availability

All data generated during this study are available in this published article.

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