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Role of Bone Morphogenetic Protein 4 in the Inflammation of the Myocardium and Vascular Tissue of Obese Mice

Ting Wu, MD, Liang Chen, MD, Qin Shen, MD, Li Wang, MD, and Gang-Jun Zong, MD, PhD

Abstract: Bone morphogenetic protein 4 (BMP4) is a proinflammatory factor. The expression of BMP4 is reduced in the adipose and enhanced in the myocardium and vascular during obesity. It is possibly involved in the process of inflammatory response of the myocardium and vascular. Obesity, often regarded as a risk factor for cardiovascular diseases, is a kind of inflammatory response. This study aimed to investigate the relationship of BMP4 with obesity and cardiovascular disease. *Ob/ob* mice were used as the experimental group, and C57BL/6 mice were used as the control group. The two groups were further divided into 2 subgroups based on the mice carrying adenovirus-encoding shRNA for BMP4 or Lac Z genes. The messenger RNA and protein levels of BMP4, interleukin-1β, and interleukin-9 were significantly higher in the myocardial tissue and aorta of *ob/ob*+ Lac Z shRNA than those in the other 3 groups, whereas the levels in the *ob/ob*+ BMP4 shRNA group were significantly decreased and comparable with those in the control groups. BMP4 is significantly upregulated in the myocardial tissue and aorta of obese mice, and this suggests that BMP4 is an risk factor involved in the local inflammatory response.

Key Words: BMP4, obesity, inflammation, cardiovascular disease

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INTRODUCTION

Obesity is one of the major causes of cardiovascular diseases (CVDs). It determines the health and the quality of life and the global burden of diseases.[1] Obesity is associated with local and systemic oxidative stress and chronic inflammation.[2,3] Increasing evidences have shown that interactions between oxidative stress and inflammatory signal pathway result in a complex and multisystem pathological state, including insulin resistance, dysfunction of endothelial cells, and increased cell apoptosis and fibrosis.[4–6] Arterial endothelial dysfunction caused by long-term obesity can eventually lead to hypertension, atherosclerosis, and other CVDs.[2–5] To date, many studies have revealed that obesity is an independent risk factor for CVDs and have highlighted the interactions between oxidative stress and chronic inflammation. However, the underlying mechanisms involved in the development of CVDs because of increased adipose tissue are still unclear.

Bone morphogenetic protein 4 (BMP4) is a growth factor of the transforming growth factor-β superfamily and was originally identified as bone-inducing proteins, but the activities of BMP4 are not restricted to bone formation, and it has been reported to induce the differentiation of pluripotent stem cells to the adipocyte lineage. The expression levels of BMP4 are higher in the adipose tissue of individuals with a lower body mass index than those with a higher body mass index.[7–9] BMP4 can activate nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, increase the production of reactive oxygen species, and reduce endothelial NO bioavailability, thus mediating the dysfunction of endothelial cells.[10–12] Our preliminary data confirmed the above idea[13]; the expression level of BMP4 decreased in the adipose tissue with an increase in the mass of adipose tissue, whereas it was increased in the myocardial tissue and aorta. The increased expression of BMP4 can further affect vascular endothelium and cardiac muscles, together promoting the inflammatory response. However, whether BMP4 is an independent risk factor for inflammatory response in myocardial tissue and aorta during obesity remains to be investigated. In this study, we studied BMP4 expression and inflammation in the myocardial tissue and aorta of obese mice by using adenovirus. The objective is to investigate the relationship of BMP4 with obesity and CVDs, which shows that BMP4 is an risk factor for the inflammation of the myocardial tissue and aorta. Our results provide evidences for effective therapeutic targets to treat CVDs caused by obesity.

MATERIALS AND METHODS

Materials

Specific pathogen-free male leptin-deficient (*ob/ob*) mice on a C57BL/6 background, along with C57BL/6 mice (weighing 18–24 g, age 4 weeks old), were provided by

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G.-J. Zong conceived the study and designed it. T. Wu performed all the experiments and drafted the manuscript. L. Chen, Q. Shen, and L. Wang performed the data analysis and created the figures. All authors read and approved the final manuscript.
Study design: A randomized controlled trial.
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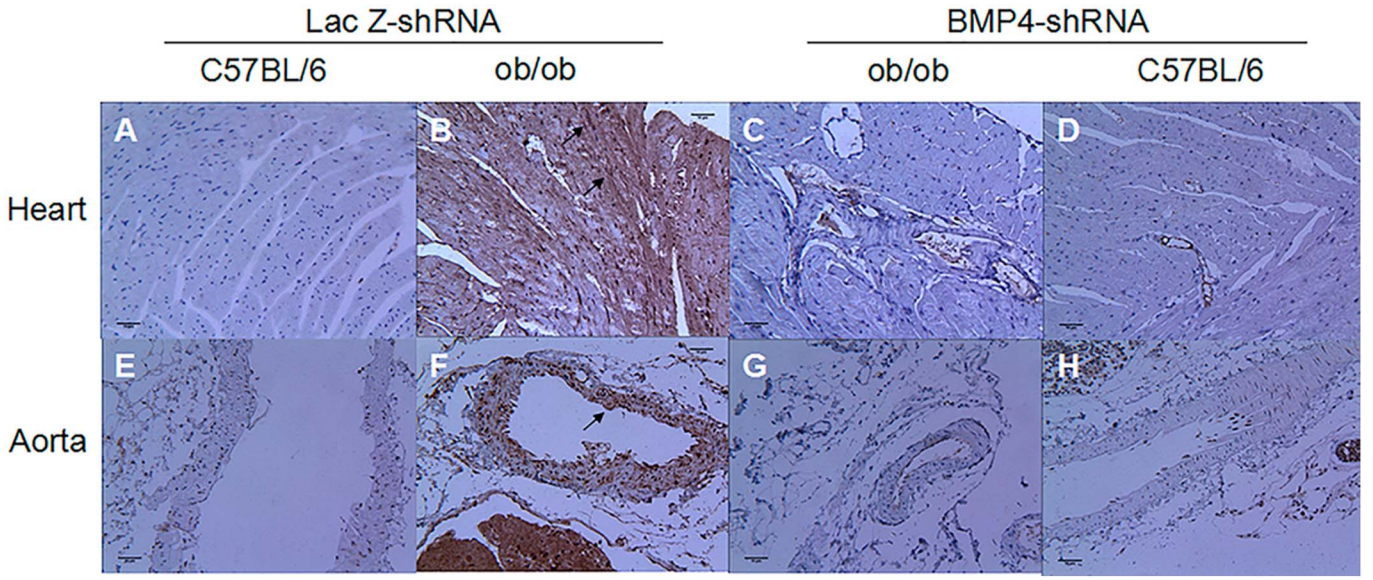

FIGURE 1. Expression of BMP4 in the heart and abdominal aorta (n = 8 for each group). BMP4 expression in the heart (A–D) and aorta (E and F) of the *ob/ob*+ BMP4 shRNA group (C and G) was significantly lower compared with that of the *ob/ob*+ Lac Z shRNA group (B and F). No obvious difference was observed between *ob/ob*+ BMP4 shRNA (C and G), C57BL/6+Lac Z shRNA (A and E), and C57BL/6+BMP4 shRNA (D and H) groups (scale bar: 20 μm).

Nanjing Qingzilan Technology (Nanjing, China). This study was approved by Animal Care and Use Committee of Anhui Medical University, and all animals received care in compliance with the Guide for the Care and Use of Laboratory Animals published in 1988 by The National Academies. Rabbit BMP4 (1:1000, ab39973), interleukin (IL)-1β (1:500, ab200478), and IL-9 (1:300, ab203386) polyclonal antibodies were purchased from Abcam (Cambridge, United Kingdom). Rabbit Smad1 (1:1000, 9743S) and phosphorylated Smad1/5/8 (p-Smad1/5/8, 1:500, 9511L) polyclonal antibodies were purchased from Cell Signaling Technology (Shanghai, China). PrimeScript RT Reagent Kit and SYBR Green I quantitative Polymerase Chain Reaction (qPCR) were purchased from Takara Bio (Otsu, Japan). Mice BMP4 shRNA interference adenovirus and Lac Z shRNA interference adenovirus were purchased from Shanghai GenePharma Technology (Shanghai, China). The sequence of BMP4 shRNA was as follows: GCCAACACTGTGAGGAGTTTC. The sequence of Lac Z shRNA was as follows: GTTCTCCGAACGTGTCACGT.

Methods

Animal Models and Groups

Ob/ob mice were used as the experimental group, and C57BL/6 mice were used for control. The two groups were further divided into BMP4 shRNA adenovirus– and Lac Z shRNA adenovirus–treated subgroups. In total, 4 groups were included in this study, each with 8 mice. Mice were maintained in the animal facility with free access to food and drink for a 12-hour dark and light period at 20–25°C and humidity of 40%–50%. The diet is standard rat chow, containing 4.5% lipids, 53% carbohydrates, and 23% proteins. Eight weeks after a normal diet, adenovirus-encoding shRNA of *BMP4* or Lac Z genes (4

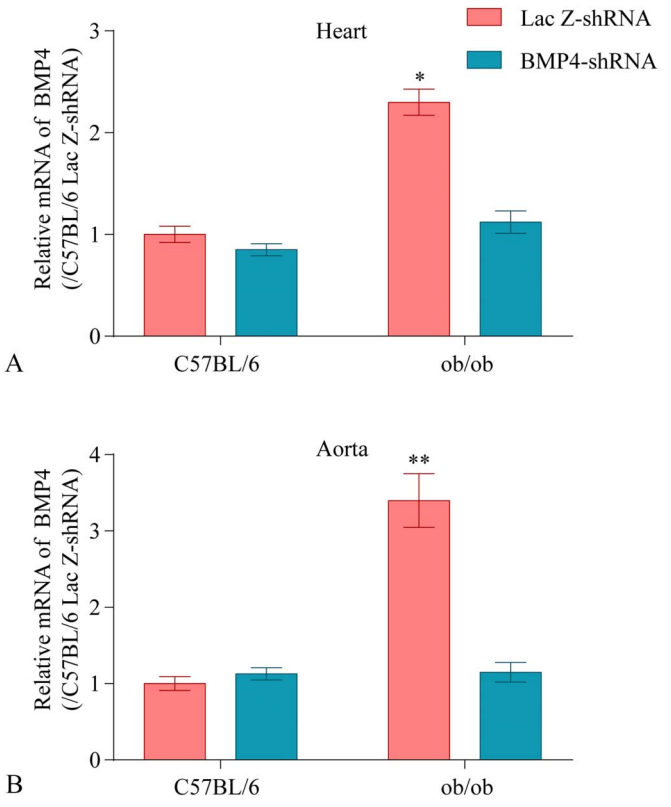

FIGURE 2. The mRNA expressions of BMP4 in the heart and aorta (n = 8 for each group). The gene expression of *BMP4* in the heart (A) and aorta (B) was remarkably reduced in the *ob/ob*+ BMP4 shRNA group than that in the *ob/ob*+ Lac Z shRNA group (**$P < 0.01$, *$P < 0.05$). No statistically significant difference was observed between the C57BL/6+Lac Z shRNA group and C57BL/6+BMP4 shRNA group ($P > 0.05$).

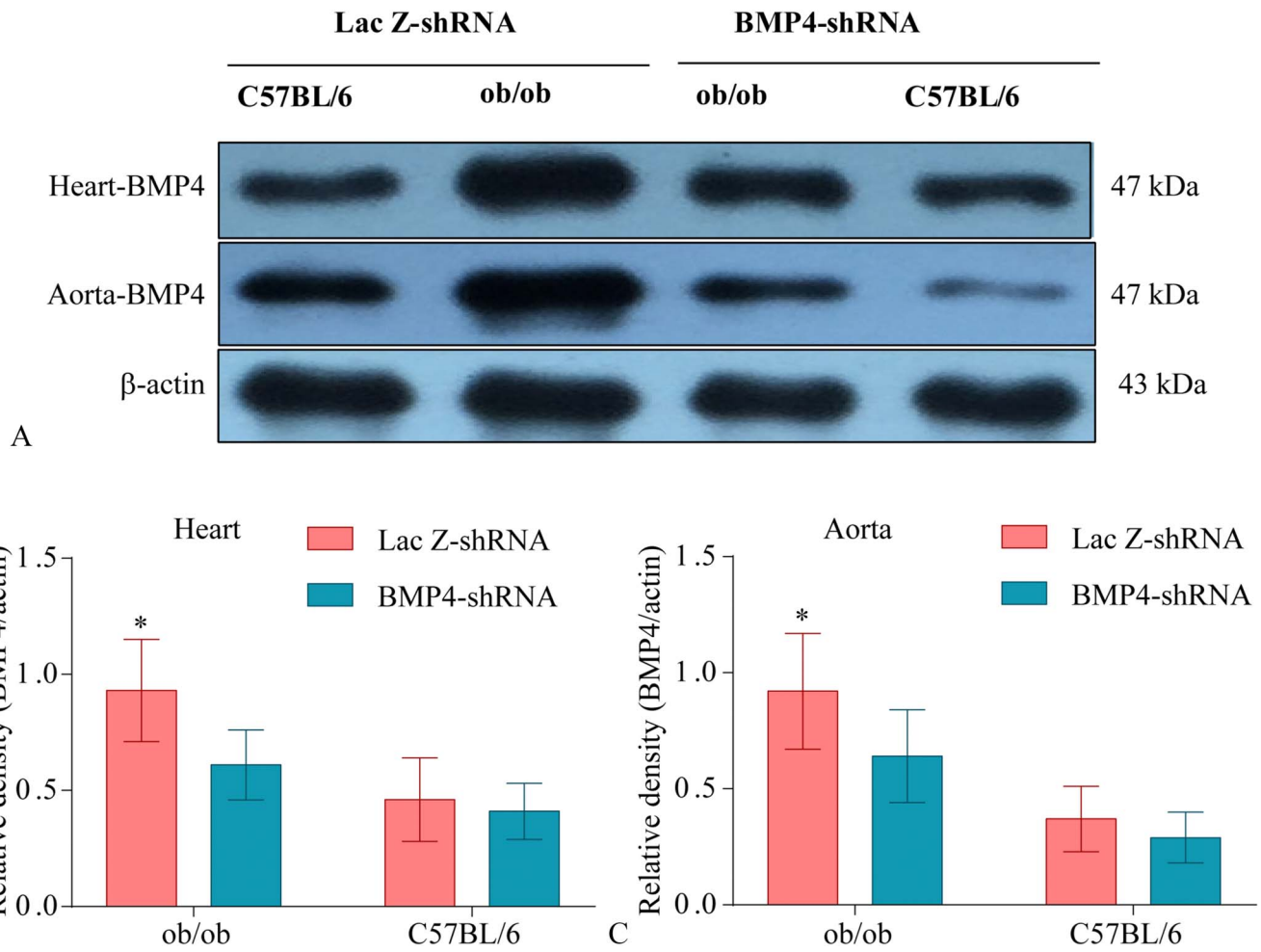

FIGURE 3. The protein expressions of BMP4 in the heart and aorta (n = 8 for each group, A). The protein expression of BMP4 was significantly reduced in the heart (B) and aorta (C) in the *ob/ob*+ BMP4 shRNA group than that in the *ob/ob*+ Lac Z shRNA group (*$P < 0.05$). No statistically significant difference was observed between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$).

mL/kg) with concentration of 1×10^9 TU/mL was intravenously injected into mice. Repeat injection was performed once a week for 4 weeks. After that, mice were sacrificed after weighting. The heart and abdominal aorta were removed for further tests.

Specimen Preparation

Tissue samples were taken from an area 5 mm to cardiac apex and 1 cm to proximal part of abdominal aorta and paraffin-embedded; the thickness of the sectioned tissues was 4 μm and stained with anti-BMP4 for immunohistochemical analyses. The remaining tissues were used for real-time polymerase chain reaction (PCR) or western blot.

Immunohistochemistry

Sections were dewaxed, rehydrated, and washed with phosphate-buffered saline (PBS) for 5 minutes, before immersion in 0.01-mol/L sodium citrate buffer (pH 6.0) and incubation in a water bath at 92–98°C for 15 minutes. Subsequently, the sections were left to cool naturally and were then washed with PBS for 5 minutes at room temperature. Sections were immersed in 3% H_2O_2 for 10 minutes and then washed with PBS. After the addition of serum diluted in PBS, sections were incubated at 37°C for 20 minutes. Anti-BMP4 antibody was diluted 1:200 in PBS containing serum, added to the sections, and incubated at 4°C overnight. The next day, the sections were washed 5 times in PBS before incubation with 1:200 diluted biotin-conjugated donkey anti-rabbit immunoglobulin G at 37°C for 30 minutes, followed by treatment with 3,3′-diaminobenzidine, and counterstaining with hematoxylin. Sections were dehydrated, cleared in xylene, and mounted under a cover slip. BMP4 expression was observed and photographed under an optical microscope.

Real-Time PCR

Total RNA was isolated from tissue specimens using TRIzol reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. Total messenger RNA (mRNA) was reverse transcribed to complementary DNA, and the mRNA expression levels of *BMP4*, *IL-1β*, and *IL-9* genes were determined by real-time PCR. The

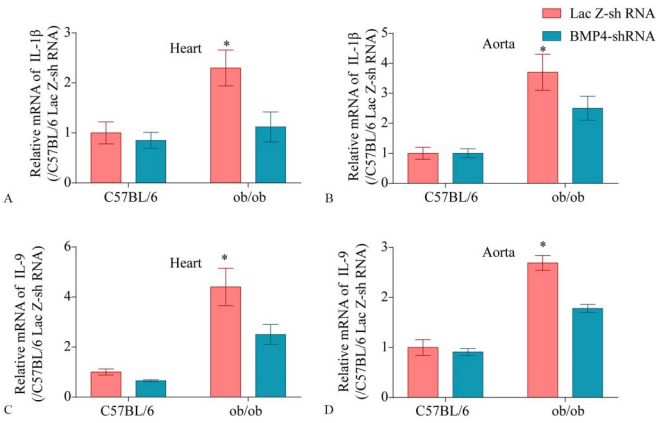

FIGURE 4. The mRNA expressions of inflammatory biomarkers in the heart and aorta (n = 8 for each group). The mRNA levels of *IL-1β* (A and B) and *IL-9* (C and D) were significantly downregulated in myocardial tissue (A and C) and aorta (B and D) of the *ob/ob*+ BMP4 shRNA group compared with those of the *ob/ob*+ Lac Z shRNA group (*$P < 0.05$). No significantly different was observed between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$).

primers (see **Supplement Table 1**, **Supplemental Digital Content 1**, http://links.lww.com/JCVP/A783) were designed with Primer 5 software and synthesized by Shanghai Biological Engineering (Shanghai, China).

Western Blot

The expression levels of *IL-1β*, *IL-9*, and *p-Smad1/5/8* genes were determined with western blot. Briefly, the tissue was homogenized and lysed using lysis buffer containing 2% (wt/vol) sodium dodecyl sulfate and 60-mM Tris HCl (pH 6.8). Supernatant was used for further analysis after centrifugation at 15,000*g*/min for 30 minutes, at 4°C. Equal amount of protein with sample loading buffer was denatured for 5 minutes at 95°C and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, followed by transfer to the polyvinylidene fluoride (PVDF) membrane. Primary antibodies were diluted in PBS/Tween containing 5% milk powder and added to the PVDF membrane for incubation at 4°C overnight. Indicated secondary antibodies were added for 2 hours at room temperature, and the proteins were finally visualized using enhanced chemiluminescence.

Statistical Analysis

All data were expressed as mean ± SD ($\bar{X} \pm s$). Statistical analysis was performed using SPSS software version 13.0. Analysis of variance was used to estimate the difference between groups, and differences between multiple groups were analyzed using one-way analysis of variance followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

RESULTS

Establishment of the Obese Mouse Model

After 12 weeks of feeding an ordinary diet, the body weight of C57BL/6 mice increased from 19.6 ± 1.47 g to 25.5 ± 1.32 g (approximately 1.3-fold), whereas that of *ob/ob* mice increased from 22.3 ± 2.05 g to 43.6 ± 2.49 g (approximately 2-fold).

The mice were considered obese when they had a 35% weight gain compared with original body weight after 13 weeks of diet intake.[14] After 12 weeks, 100% mice in our experimental group fit the criteria of obesity.

Protein and mRNA Levels of BMP4 in Different Groups

The mice were assigned to 4 groups: C57BL/6+Lac Z shRNA; C57BL/6+BMP4 shRNA; *ob/ob*+ Lac Z shRNA; and *ob/ob*+ BMP4 shRNA. Immunohistochemistry staining was performed to evaluate the expression of BMP4 in myocardial tissue and aorta. Results reveal that the expression of BMP4 in the myocardial tissue and abdominal aorta of the *ob/ob*+ BMP4 shRNA group was significantly lower compared with that of the *ob/ob*+ Lac Z shRNA group. In addition, no statistically significant difference was observed between C57BL/6+Lac Z shRNA and C57BL/6+BMP4 shRNA. Of note, the levels of BMP4 in C57BL/6 mice showed a significant decrease compared with that in the *ob/ob*+ Lac Z shRNA group (Fig. 1).

After this, real-time PCR was performed to detect *BMP4* mRNA levels in cardiovascular tissue of different groups. Results reveal that the *BMP4* mRNA levels showed a significant decrease in the *ob/ob*+ BMP4 shRNA group compared with that in the *ob/ob*+ Lac Z shRNA group ($P < 0.05$). However, no significant difference of *BMP4* mRNA levels was observed between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$) (Fig. 2).

Western blot demonstrated that treatment with BMP4 shRNA markedly reduced the expression of *BMP4* in myocardial tissue and aorta in *ob/ob* mice ($P < 0.05$). The protein levels of BMP4 in the *ob/ob*+ BMP4 shRNA group were not significant difference than those in the C57BL/6+Lac Z shRNA and C57BL/6+BMP4 shRNA groups ($P > 0.05$) (Fig. 3).

Protein and mRNA Levels of Proinflammatory Cytokines in Different Groups

We hypothesized that BMP4 was an independent risk factor for myocardial and artery inflammation in obese mice. To test this hypothesis, we measured the levels of 2 proinflammatory factors, *IL-1*β and *IL-9*, by real-time PCR and western blot. The mRNA levels of *IL-1*β and *IL-9* were significantly downregulated in myocardial tissue and aorta of the *ob/ob*+ BMP4 shRNA group compared with those of the *ob/ob*+ Lac Z shRNA group ($P < 0.05$). In addition, the mRNA levels of *IL-1*β and *IL-9* were not significantly different in myocardial tissue and aorta between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$) (Fig. 4).

Western blot results demonstrated that the protein levels of IL-1β and IL-9 in myocardial tissue and aorta were markedly lower in the *ob/ob*+ BMP4 shRNA group than those in the *ob/ob*+ Lac Z shRNA group ($P < 0.05$) (Fig. 5).

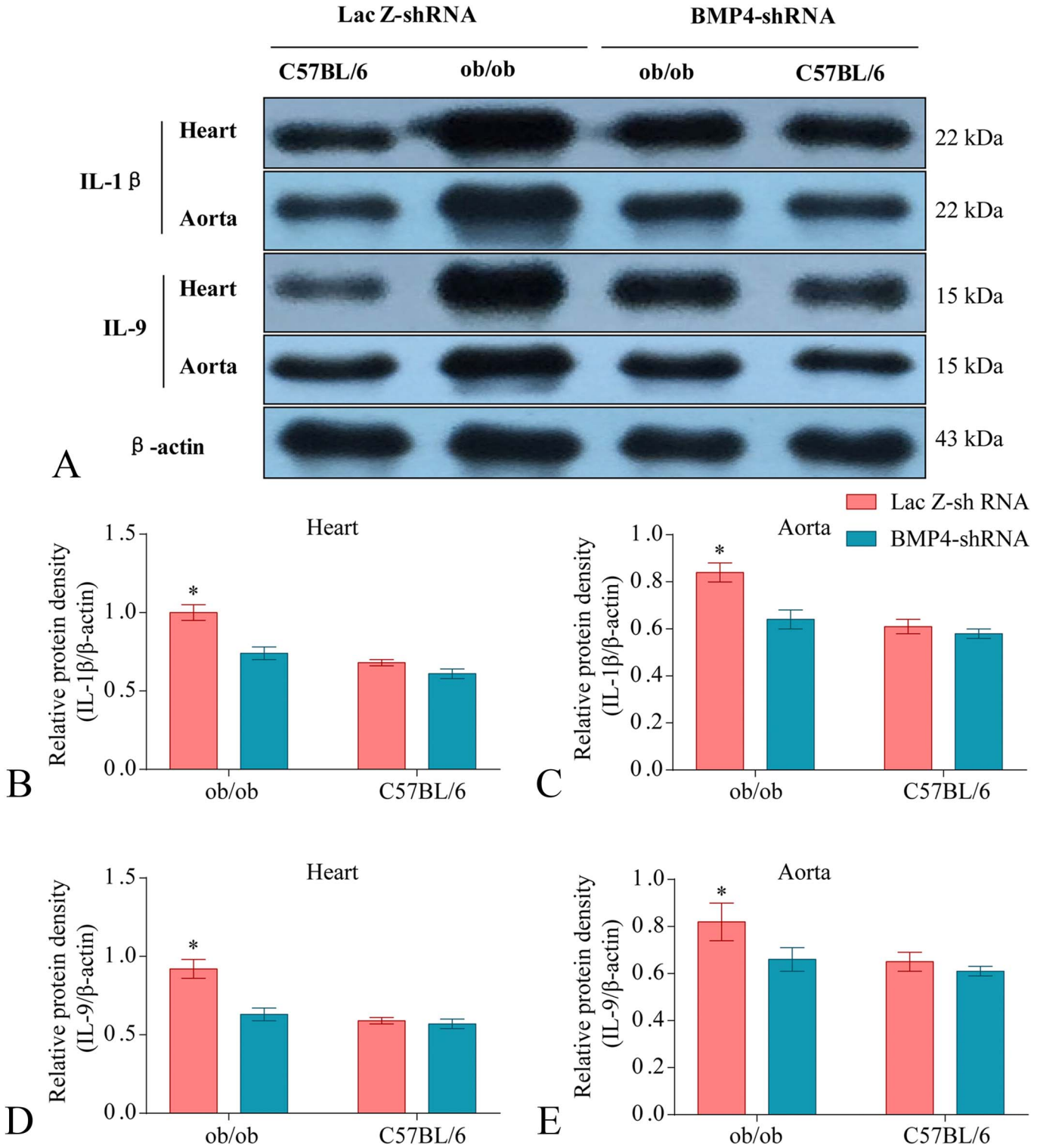

FIGURE 5. The protein expressions of inflammatory biomarkers in the heart and aorta (n = 8 for each group, A). The protein levels of IL-1β (B and C) and IL-9 (D and E) in myocardial tissue (B and D) and aorta (C and E) in the *ob/ob*+ BMP4 shRNA group were markedly lower than those in the *ob/ob*+ Lac Z shRNA group (*$P < 0.05$). No statistically significantly different was observed between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$).

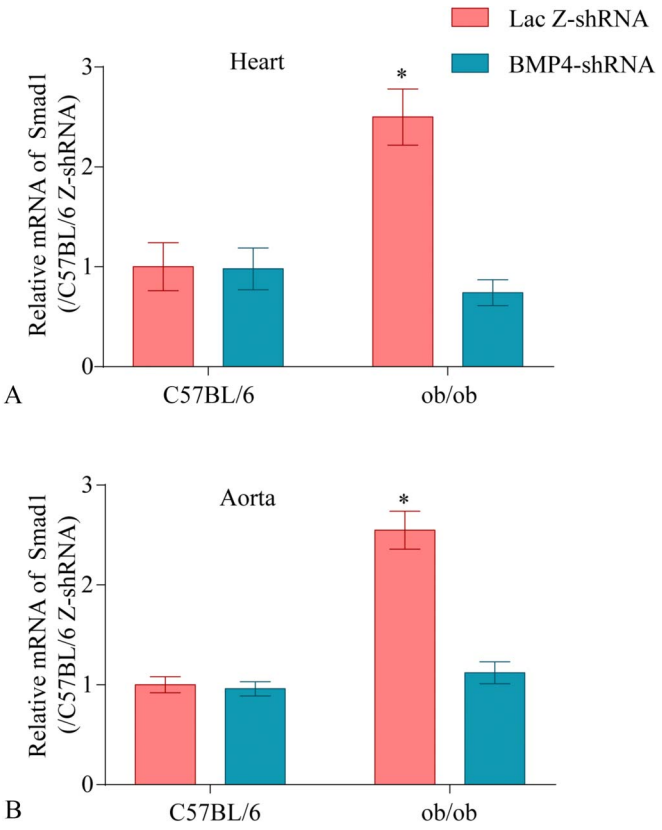

FIGURE 6. The mRNA expression of Smad1 in the heart and aorta (n = 8 for each group). The mRNA levels of *Smad1* in myocardial tissue (A) and aorta (B) were significantly reduced in the *ob/ob*+ BMP4 shRNA group compared with that in the *ob/ob*+ Lac Z shRNA group (*$P < 0.05$).

Protein and mRNA Levels of Genes in BMP4 Signaling Pathway

It has been reported that BMP4 regulates the inflammatory process by rapid phosphorylation of *Smad1*.[15,16] To confirm the relationship of BMP4 expression and inflammation of myocardial tissue and aorta, we studied the expression levels of genes involved in BMP4 signaling pathway. The protein and mRNA levels of *Smad1* in myocardial tissue and aorta were significantly reduced in the *ob/ob*+ BMP4 shRNA group compared with that in the *ob/ob*+ Lac Z shRNA group ($P < 0.05$) (Figs. 6 and 7). The phosphorylation of *Smad1/5/8* was in consistent the results of *Smad1* ($P < 0.05$) (Fig. 7). No significant difference was observed in *ob/ob*+ BMP4 shRNA, C57BL/6+BMP4 shRNA, and C57BL/6+Lac Z shRNA groups ($P > 0.05$) (Figs. 6 and 7).

DISCUSSION

In this study, we used *ob/ob* mice, which lack leptin and become obese under normal dietary conditions, enabling us to exclude the effects of a high-fat diet on the cardiovascular system. We successfully established the mice model for obesity and showed that tissue concentration of BMP4 was elevated in the heart and artery tissue of obese mice and that local inflammation in these mice was mediated by BMP4. We also demonstrated that the inflammatory response could be ameliorated by downregulation of BMP4 in the heart and artery tissues.

Obesity promotes local inflammation of cardiovascular tissues, which may be a major cause of high incidence of CVDs in obese people.[17,18] Many studies have investigated local and systemic inflammation in obese people, whereas reports about the exact effect of only the increase of adipose tissues on the cardiovascular system are still limited. Our previous study demonstrated that although the protein level of BMP4 decreased with the increase of fat tissue, no significant changes were observed in the *BMP4* mRNA level.[13] Therefore, we propose that BMP4 relocates from adipose tissue into the blood, leading to its decreased level in adipose tissues and increased level in myocardial tissue and aorta. Thus, the increased BMP4 causes local inflammatory response in myocardial tissue and aorta and leads to the occurrence of CVDs. Results obtained in this study showed that the protein level of BMP4 was indeed enhanced with increase of adipose tissue in myocardial tissue and aorta. IL-1β and IL-9 are important inflammatory factors and play key roles in the pathogenesis of atherosclerosis. Many studies have confirmed that IL-1β and IL-9 levels were found to be increased in plasma and carotid plaques of patients with carotid and coronary atherosclerosis.[19,20] In this study, the protein and mRNA levels of IL-1β and IL-9 increased in obese mice, promoting inflammation in myocardial tissue and aorta. Smad1 is the immediate downstream molecules of BMP receptors and plays a central role in BMP signal transduction.[21] We further verified that BMP4 enhanced inflammation through Smad1 pathway. In fact, CVDs are caused by chronic, low-grade, inflammation.[22] Therefore, elevated inflammatory response is related to increased incidence of hypertension, atherosclerosis, and other CVDs. Taken together, our findings demonstrate that BMP4 mediates inflammation of cardiovascular tissues and bridges increased adipose tissue and enhanced cardiovascular inflammatory response.

Obesity is a multifactorial and multisystemic response of the body, which is characterized by local and systemic chronic inflammation.[2–5] It remains unclear whether increased inflammation in myocardial tissue and aorta is mediated by multifactors. To exclude the effect of other factors and investigate whether BMP4 is an independent risk factor for cardiovascular inflammation in obesity, we used adenovirus to downregulate the expression of *BMP4*. The expression level of *BMP4* reduced to the baseline in obese mice. Interference in *BMP4* expression had no effect on the protein and mRNA levels of IL-1β and IL-9 in the control C57BL/6 mice, which suggests that physiological amounts of BMP4 do not necessarily cause heart and arterial inflammation. However, when BMP4 levels decreased in obese mice, the amounts of proinflammatory cytokines, IL-1β and IL-9, were attenuated and were comparable with the levels in control mice, suggesting that the inflammation was mediated by BMP4. Together, the above results strongly demonstrate BMP4 as an independent risk factor of heart and arterial inflammation in obese mice.

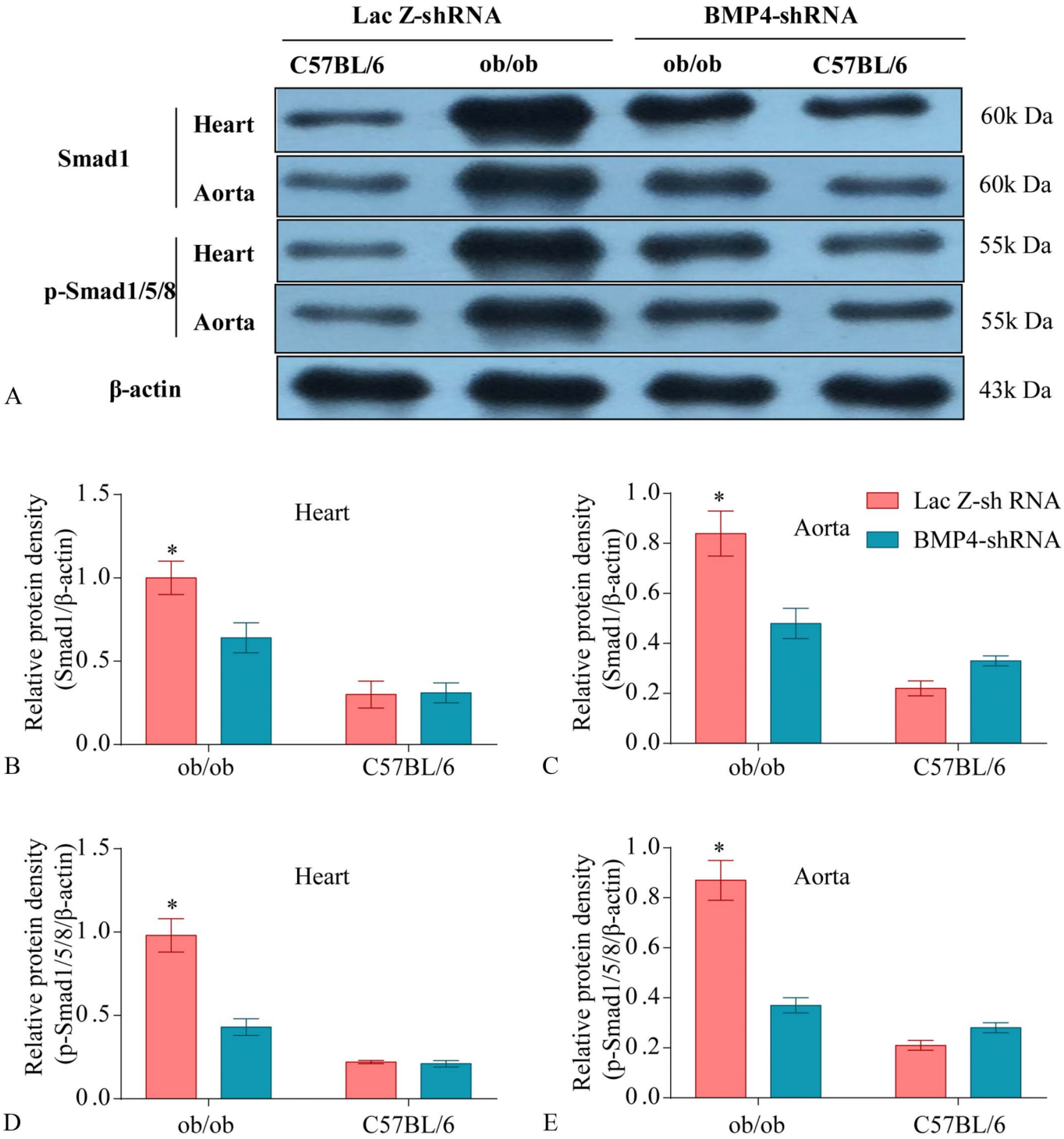

FIGURE 7. The protein expressions of Smad1 and p-Smad1/5/8 in the heart and aorta (n = 8 for each group, A). The protein expressions of Smad1 (B and C) and p-Smad1/5/8 (D and E) were significantly reduced in the heart (B and D) and aorta (C and E) in the *ob/ob*+ BMP4 shRNA group compared with that in the *ob/ob*+ Lac Z shRNA group (*$P < 0.05$). No statistically significantly different was observed between *ob/ob*+ BMP4 shRNA, C57BL/6+Lac Z shRNA, and C57BL/6+BMP4 shRNA groups ($P > 0.05$).

In conclusion, we verified BMP4 as an independent risk factor for heart and arterial inflammation in obesity. BMP4 was shown to play a major role in obesity-induced CVDs, which might be a new clinical target in preventing and treating CVDs of obese patients. Because the pathogenesis of obesity and CVDs is complex, whether adipose tissue can directly regulate the expression of BMP4 in cardiovascular tissues remains to be further investigated.

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