

Ghrelin receptor deficiency aggravates atherosclerotic plaque instability

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TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Animals
 - 3.2. Measurement of plasma lipid levels
 - 3.3. Oil-red staining and immunohistochemistry
 - 3.4. Western blot analysis
 - 3.5. Statistical analysis
4. Results
 - 4.1. Plasma lipid levels
 - 4.2. Atherosclerotic plaque
 - 4.3. Histology findings of T cell, macrophage, smooth muscle cell and vascular endothelial cell
 - 4.4. Expression of VCAM-1 and ICAM-1 protein
5. Discussion
6. Conclusion
7. Acknowledgements
8. References

1. ABSTRACT

Ghrelin has been found to be associated with anti-inflammatory effects, inhibition of atherosclerotic plaque formation and plaque stability in the cardiovascular system. We investigated whether ghrelin affected atherosclerotic plaque and inflammation found in atherosclerosis. We crossed ghrelin receptor knockout mice (GHSR^{-/-}) and low-density lipoprotein receptor-null (low- LDLR^{-/-}) mice. In this model, serum lipid levels, atherosclerotic plaque on the aortic arches, and expression of ICAM-1 and VCAM-1, T cells, macrophages, and smooth muscle cells of atherosclerotic plaque were observed. Although serum lipid levels and atherosclerotic plaque in aortic arches were not significantly different between GHSR^{+/+}/LDLR^{-/-} and GHSR^{-/-}/LDLR^{-/-} mice, ICAM-1 and VCAM-1 protein expression in atherosclerotic plaques were increased in GHSR^{-/-}/LDLR^{-/-} mice compared with GHSR^{+/+}/LDLR^{-/-} mice.

T cells and macrophages were increased, while smooth muscle cells of atherosclerotic plaques were less in GHSR^{-/-}/LDLR^{-/-} mice than that in GHSR^{+/+}/LDLR^{-/-} mice. In conclusion, ghrelin receptor deficiency aggravates atherosclerotic plaque instability and vascular inflammation. This information will provide novel avenues for the treatment of patients with atherosclerosis.

2. INTRODUCTION

Ghrelin is a gastric 28 amino acid peptide hormone and has been proposed as the endogenous ligand for growth hormone secretagogue receptor (GHSR) (1). In addition to the stomach, ghrelin and its receptor GHSR are distributed in the cardiovascular system, and also are highly expressed in the heart, atria, and vascular system, suggesting that ghrelin and GHSR play important roles in the cardiovascular system (2).

A mouse model of coronary atherosclerosis showed that GSHR density in the cardiovascular system was 30%-40% higher than in control mice (3). GSHR binding experiments also showed its wide distribution in the human cardiovascular system, and further found an increased density of GSHR in coronary atherosclerotic plaques (4). Thus, the role of ghrelin and GSHR in cardiovascular disease has been an area of recent focus.

Atherosclerosis is a chronic inflammatory disease, in which endothelial injury leads to a chronic inflammatory fibroproliferative response. Both adaptive and innate immune responses, especially macrophages and T cells, are involved in arterial plaque formation (5–8). Plasma ghrelin levels are associated with coronary microvascular and endothelial dysfunction in peritoneal dialysis patients (9). An *in vitro* study demonstrated a protective effect of ghrelin in porcine coronary arteries, by inhibiting endothelial dysfunction (10). Exogenous ghrelin demonstrates an anti-inflammatory effect, by inhibiting the release of adhesion molecules, chemokines, and monocyte adhesion in tumor necrosis factor- α -induced human umbilical vein endothelial cells (HUVECs) (11). In our previous study, we also found that exogenous ghrelin inhibits CD40 expression via cytokine-mediation in HUVECs (12). We furthermore found that ghrelin inhibits proliferation and apoptosis of vascular smooth muscle cell, which may in turn inhibit atherosclerotic plaque formation and promote plaque stability (13). In LDL-receptor (LDLR $^{-/-}$) deficient mice, ghrelin vaccination can decrease plasma MCP-1 levels, resulting in inhibition of inflammation (14). Therefore, ghrelin may play a protective role in atherosclerosis by inhibiting endothelial inflammation and plaque formation. Recently, Habegger *et al.* (15) reported that endogenous GSHR-mediated signaling on the cardiovascular system minimally impacts atherosclerotic plaque progression, as based on a GSHR $^{-/-}$ LDLR $^{-/-}$ mouse model of atherosclerosis. However, this particular study did not demonstrate a direct relationship between GSHR and vascular inflammation or atherosclerotic plaque components in atherosclerosis.

In the present study, we generated a GSHR $^{-/-}$ LDLR $^{-/-}$ mouse model of atherosclerosis and analyzed GSHR-dependent effects on inflammation in atherosclerotic plaque. We analyzed lesion size, plasma lipid levels, and the expression of CD4 $^{+}$ T cells, macrophages, and adhesion molecules in a GSHR $^{-/-}$ and LDLR $^{-/-}$ mouse

model. We found that ablation of GSHR improved atherosclerotic plaque instability.

3. METHODS

3.1. Animals

GSHR-deficient mice were obtained from the Shanghai Research Center for Biomedical Organisms (Shanghai, China). LDLR-deficient mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Mice from both lines were bred in our facilities and maintained on a C57/B6 background. LDLR knockout (LDLR $^{-/-}$) mice of both sexes were crossed with GSHR $^{-/-}$ mice to generate a double-heterozygous generation. Double-heterozygous mice were inbred to produce mice deficient in both LDLR and GSHR (GSHR $^{-/-}$ LDLR $^{-/-}$) or LDLR only (GSHR $^{+/+}$ LDLR $^{-/-}$). DNA was extracted from tail snips and analyzed by PCR to establish genotypes. GSHR $^{-/-}$ LDLR $^{-/-}$ genotyping used the following primers: 5'- AAT CCA TCT TGT TCA ATG GCC GAT C-3', 5'- CCA TAT GCA TCC CCA GTC TT-3' and 5'- GCG ATG GAT ACA CTC ACT GC-3'. Resultant wild-type, flox, and delta flox (deleted) allele bands were 167, 167, and 350 base pairs (bp), respectively. All animals were housed on a 12:12 h light–dark cycle at 22° C. At 4 weeks of age, 12 male mice in each of the wild type, GSHR $^{-/-}$ LDLR $^{+/+}$, and GSHR $^{+/+}$ LDLR $^{-/-}$ groups, were fed a diet enriched with saturated fat (45% wt/wt milk fat; Slac Laboratory Animal, Shanghai, China) for 18 weeks. All study procedures were approved by the Institutional Animal Care and Use Committee of Shanghai Tongji University.

3.2. Measurement of plasma lipid levels

Blood samples (1 mL) from the right ventricle were analyzed individually from all animals. Plasma total cholesterol (TC), triglycerides, high-density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured with the colorimetric method (Cholesterol Gen.2, Triglycerides GPO-PAP, LDL-Cholesterol plus 2nd generation; Roche Diagnostics GmbH, Oberbayern, Germany).

3.3. Oil-red staining and immunohistochemistry

Tissue specimens were taken from aortic arches of mice, transferred to a 10% neutral buffer, formalin fixed, and paraffin embedded. Deparaffinized sections (6 μ m) were stained with oil-red and analyzed by light microscopy. The degree of atherosclerosis was quantified using en-face analysis of the aortic intima of the aortic arch and thorax, as described previously (16).

Table 1. Plasma lipid levels

Plasma lipid levels	Wide type mice	GSHR+/+LDLR ^{-/-} mice	GSHR ^{-/-} LDLR ^{-/-} mice	p-value (between three groups)	p-value (GSHR+/+LDLR ^{-/-} vs. GSHR ^{-/-} LDLR ^{-/-} mice)
TC (mmol/L)	3.2.35±0.5.74	18.5.25±2.9.51	19.2.55±3.2.75	>0.0.1	0.3.29
triglycerides	1.3.50±0.2.30	5.9.08±0.5.19	4.9.27±0.9.65	>0.0.1	0.0.56
HDL	1.6.45±0.1.49	0.9.35±0.1.82	0.8.60±0.2.10	>0.0.1	0.5.85
LDL	2.2.73±0.8.32	15.8.18±2.1.68	16.1.08±2.6.84	>0.0.1	0.1.08
Total cholesterol: TC; high-density lipoprotein: HDL; low-density lipoprotein: LDL.					

For immunohistochemistry, formalin-fixed, paraffin-embedded tissue was cut into 6-µm-thick sections. Sections were blocked with normal goat serum (Abcam Inc., USA). Membranes were then incubated with the following primary antibodies: Mac-3 (Li-Cor Biosciences, Lincoln, NE, USA; catalog No: PA3-821A), CD4 (Li-Cor), α-actin (Li-Cor), vascular cell adhesion molecule 1 (VCAM-1), and intercellular cell adhesion molecule-1 (ICAM-1), for 1 h at room temperature. The resulting slides were incubated in a humidity chamber for 10 min with biotinylated secondary antibody and streptavidin, with intervening and subsequent rinses in PBS three times for 5 min. 3′3-diaminobenzidine-tetrahydrochloride was applied as a chromogen for 5 min. Sections were counterstained in hematoxylin for 1 min. Image Pro software (Media Cybernetics, Silver Springs, MD, USA) was used to analyze the aortic area. The area of positive staining was analyzed as previously described (17).

3.4. Western blot analysis

Tissues in the aortic arches of mice were extracted in RIPA lysis buffer. Extracts (30 µg protein) were resolved by SDS-PAGE (7.5. % wt/vol) and transferred electrophoretically to PVDF membranes. After blocking with TBS-Tween 20 (TBST) containing 5% skim milk, the membranes were incubated with the following antibodies overnight at 4° C: VCAM-1, ICAM-1 (R&D), and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were then incubated with goat anti-rabbit or anti-mouse antibodies (Li-Cor) for 1 h. Finally, blots were developed with a chemiluminescent reagent (Cell Signaling Technology Inc., Danvers, MA, USA).

3.5. Statistical analysis

SPSS version 13.0. (SPSS Inc., Chicago, IL, USA) statistical software was used for statistical analysis. Group data were expressed as the mean ± standard deviation (SD) and analyzed by one-way

analysis of variance, the Student–Newman–Keuls test (comparisons between multiple groups), or unpaired Student's t test (between groups) as appropriate. A p-value of less than 0.0.5 was considered to be statistically significant.

4. RESULTS

4.1. Plasma lipid levels

The plasma levels of TC, triglycerides, and LDL in both GSHR^{-/-}LDLR^{-/-} (TC: 19.2.55±3.2.75 mmol/L; triglycerides: 4.9.27±0.9.65; LDL: 16.1.08±2.6.84) and GSHR^{+/+}LDLR^{-/-} mice (TC: 18.5.25±2.9.51 mmol/L; triglycerides: 5.9.08±0.5.19; LDL: 15.8.18±2.1.68) were significantly higher than in wt mice (TC: 3.2.35±0.5.74 mmol/L; triglycerides: 1.3.50±0.2.30; LDL: 2.2.73±0.8.32; all p-values <0.0.1), while HDL levels in both GSHR^{-/-}LDLR^{-/-} (0.8.60±0.2.10) and GSHR^{+/+}LDLR^{-/-} (0.9.35±0.1.82) mice were decreased compared with wt mice (1.6.45±0.1.49; all p-values <0.0.1). There was no significant difference between GSHR^{-/-}LDLR^{-/-} and GSHR^{+/+}LDLR^{-/-} mice with regards to TC, triglyceride, HDL, or LDL levels (Figure 1; Table 1).

4.2. Atherosclerotic plaque

Mice of both GSHR^{-/-}LDLR^{-/-} and GSHR^{+/+}LDLR^{-/-} genotypes displayed considerable arteriosclerotic lesions on their aortic arches. In GSHR^{-/-}LDLR^{-/-} mice, lesion areas covered 56.4.2±4.9.6% of the overall vessel wall, compared with 52.4.7±6.7.1% in GSHR^{+/+}LDLR^{-/-} mice. No significant difference between GSHR^{-/-}LDLR^{-/-} mice and GSHR^{+/+}LDLR^{-/-} mice was found with regards to arteriosclerotic plaque formation (Figure 2).

4.3. Histology findings of T cell, macrophage, smooth muscle cell and vascular endothelial cell

Both CD4⁺ T cells and MAC-3⁺ macrophages were observed in both

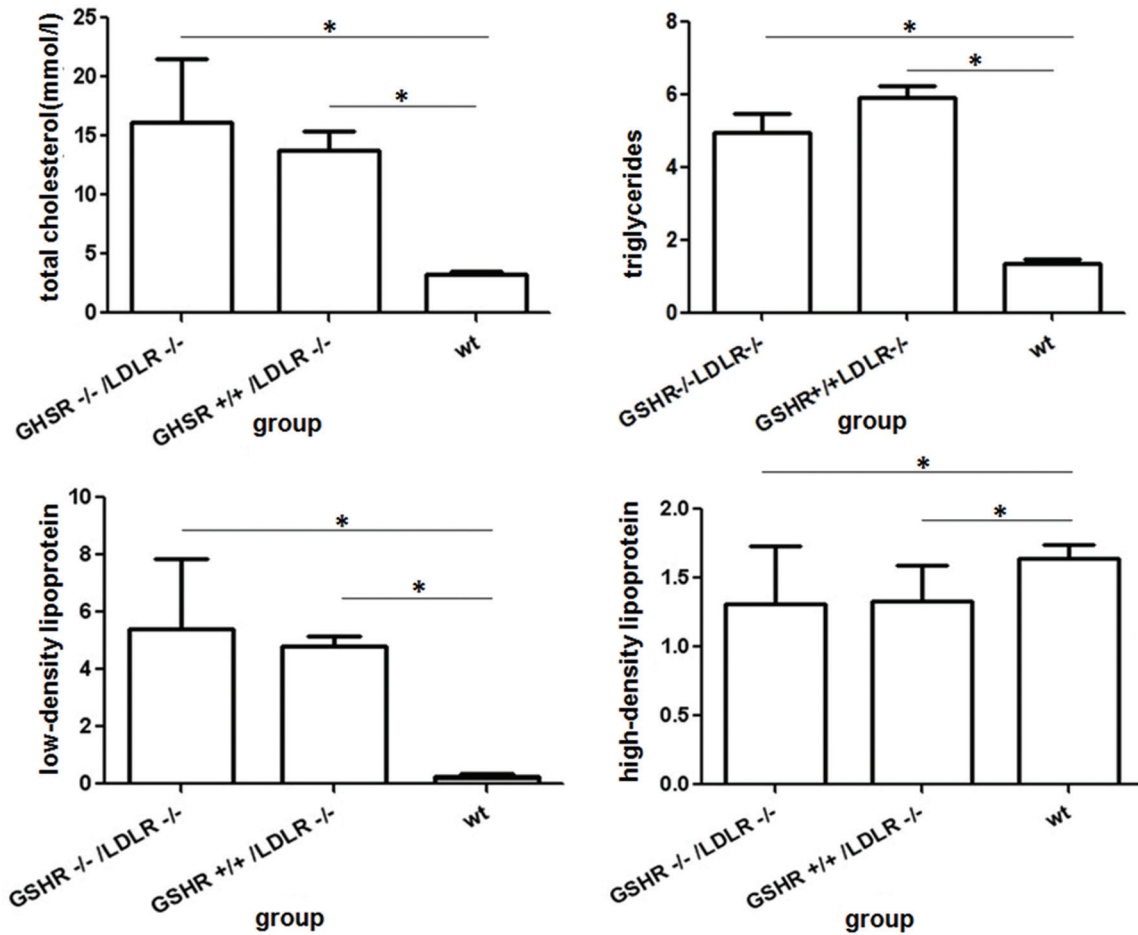


Figure 1. Plasma levels of TC, triglycerides, LDL, and HDL. The plasma levels of TC, triglycerides, and LDL in both GSHR^{-/-}/LDLR^{-/-} and GSHR^{+/+}/LDLR^{-/-} mice were significantly higher than in wt mice, while HDL levels in both GSHR^{-/-}/LDLR^{-/-} and GSHR^{+/+}/LDLR^{-/-} mice were decreased compared with wt mice. *: $p < 0.01$.

GSHR^{-/-}/LDLR^{-/-} and GSHR^{+/+}/LDLR^{-/-} mice on immunohistochemical analysis, especially in the arterial intima. Moreover, the area of infiltration of CD4⁺ T and MAC-3⁺ macrophages in GSHR^{-/-}/LDLR^{-/-} mice (CD4⁺ T: $5.9.6 \pm 0.7.4\%$; MAC-3⁺ macrophages: $38.5.4 \pm 2.1.0\%$) was larger than in GSHR^{+/+}/LDLR^{-/-} mice (CD4⁺ T: $4.0.2 \pm 0.5.3\%$; MAC-3⁺ macrophages: $33.1.7 \pm 2.7.6\%$ vs. CD4⁺ T: $p < 0.0.1$; MAC-3⁺ macrophages: $p < 0.0.5$; Figures 3,4).

The amount of aortic α -actin⁺ smooth muscle cells in GSHR^{-/-}/LDLR^{-/-} mice was decreased compared to GSHR^{+/+}/LDLR^{-/-} mice. The area of the smooth muscle cells in the overall vessel wall was $25.7.7 \pm 3.5.7\%$ in GSHR^{-/-}/LDLR^{-/-} mice, versus

$19.5.2 \pm 3.0.2\%$ in GSHR^{+/+}/LDLR^{-/-} mice ($p < 0.0.1$; Figures 3,4).

Most VCAM-1⁺ cells and ICAM-1⁺ cells were expressed in vascular endothelial cells. The infiltrating area of VCAM-1⁺ and ICAM-1⁺ cells in GSHR^{-/-}/LDLR^{-/-} mice (VCAM-1⁺ cells: $22.1.6 \pm 1.2.8\%$; ICAM-1: $21.3.7 \pm 1.9.4\%$) was larger than in GSHR^{+/+}/LDLR^{-/-} mice (VCAM-1⁺ cells: $19.7.1 \pm 2.0.4\%$; ICAM-1: $6.4.2 \pm 2.5.6\%$) (for VCAM-1⁺ cells: $p < 0.0.5$; for ICAM-1 cells: $p < 0.0.1$; Figures 3,4).

4.4. Expression of VCAM-1 and ICAM-1 protein

In the GSHR^{-/-}/LDLR^{-/-} group, both VCAM-1 ($1.2.1 \pm 0.2.3$) and ICAM-1 ($1.2.8 \pm 0.3.1$)

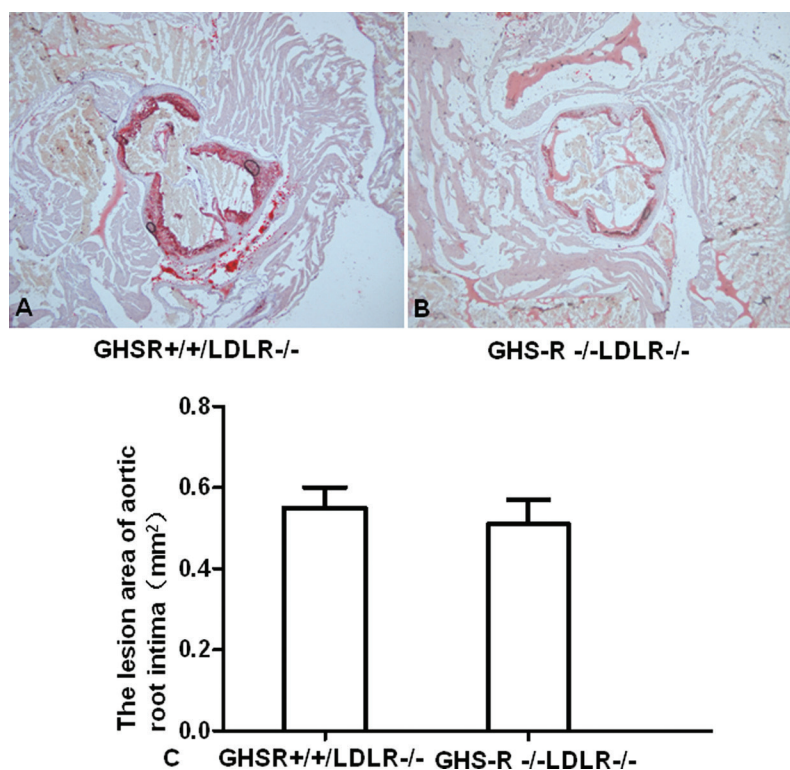


Figure 2. Oil-red staining of aortic arches. Both GSHR-/-LDLR-/- and GSHR+/+/LDLR-/- mice displayed considerable arteriosclerotic lesions on their aortic arches. Although no significant differences in lesion areas were found between groups, there was an increased trend noted in GSHR-/-LDLR-/- mice compared with GSHR+/+/LDLR-/- mice.

protein expression levels were significantly increased compared with GSHR+/+/LDLR-/- mice (VCAM-1: $0.7.5\pm0.1.6$; ICAM-1: $0.7.9\pm0.2.1$; both p-values $<0.0.1$; Figure 5).

5. DISCUSSION

We demonstrated that the expression of both CD4+ T cells and MAC-3+ macrophages is higher, while that of smooth muscle cells is lower in GSHR-/-LDLR-/- mice compared with GSHR+/+/LDLR-/- mice. The expression of VCAM-1+ and ICAM-1+ cells and VCAM-1 and ICAM-1 protein expression are increased in GSHR-/-LDLR-/- mice compared with GSHR+/+/LDLR-/- mice. However, there was no significant difference in plasma lipid levels and atherosclerotic plaque between GSHR-/-LDLR-/- and GSHR+/+/LDLR-/- mice. These findings suggest that ghrelin receptor deficiency aggravates atherosclerotic plaque instability and vascular inflammation in low-density lipoprotein receptor-null mice.

We also found considerable arteriosclerotic lesions in the aortic arches of GSHR-/-LDLR-/- mice, confirming that endothelial injury occurred in this location. Moreover, we found increased expression of adhesion molecules VCAM-1 and ICAM-1, CD4+ T cells, and MAC-3+ macrophages in these aortas, and decreased smooth muscle cells, as compared with GSHR+/+/LDLR-/- mice. Since atherosclerotic plaque instability is primarily due to its cellular components and spatial composition (18,19), our findings suggest that atherosclerotic plaque may be more unstable when ghrelin receptor is deficient. A recent study by Habeqger *et al.* (15) investigated the role of ghrelin receptor deficiency in atherosclerotic plaque. Although inflammatory cytokines or cells in atherosclerotic plaque were not analyzed, atherosclerotic plaque size was evaluated and no difference between GSHR-/-LDLR-/- and GSHR+/+/LDLR-/- mice was found. We confirmed this finding and furthermore found that plasma levels of TC, triglycerides, HDL, and LDL did not differ between mice with or without a GHSR deficiency. This

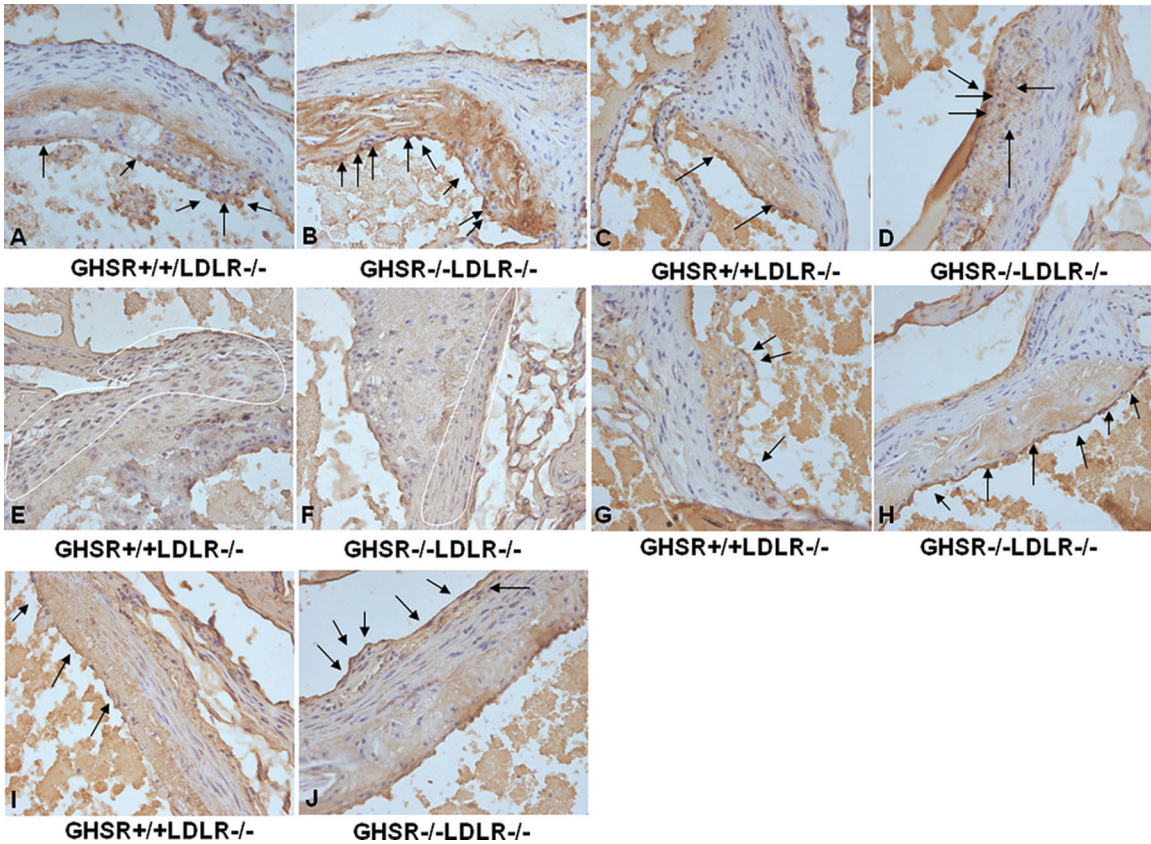


Figure 3. Immunohistochemistry of CD4+ T cells, MAC-3+ macrophages, α -actin+ smooth muscle cells, VCAM-1+ cells, and ICAM-1+ cells in aortic arches of GSHR^{-/-}LDLR^{-/-} mice and GSHR^{+/+}LDLR^{-/-} mice. A and B: CD4+ T cells; C and D: MAC-3+ macrophages; E and F: smooth muscle cells (the area of smooth muscle cells is outlined); G and H: VCAM-1+ cells; I and J: ICAM-1+ cells. Positive cells are indicated by arrows.

suggests that GHSR deficiency may be the factor to affect the components of atherosclerotic plaque, and that differences in atherosclerotic plaque composition may be the direct result of GHSR deficiency, without being affected by plasma lipid levels.

Interestingly, components of atherosclerotic plaque that are increasingly expressed in GHSR deficient mice include CD4+ T cells, macrophages, VCAM-1, and ICAM-1, which are proinflammatory factors. Since endothelial inflammation is the determinant of plaque stability (18, 19), our results suggest that GHSR deficiency may aggravate vascular inflammation involved in both adaptive and innate immune responses in atherosclerosis. This finding is consistent with previous studies on the role of ghrelin in atherosclerosis. Chow *et al.* (20) found that vascular inflammation was attenuated when cells were pretreated with ghrelin via ghrelin receptor

binding. Along with an anti-inflammatory effect, ghrelin is involved in post-infarct myocardial remodeling and improvement of cardiac function (21). An *in vitro* study of rat cardiac microvascular endothelial cells showed that ghrelin inhibits homocysteine-induced dysfunction and inflammatory response (22). A very recent study reported that ghrelin has a protective effect on hypoxia-mediated human pulmonary artery endothelial cells, via the phosphoinositide 3-kinase (PI3K)/Akt pathway (23). We confirmed the anti-inflammatory effect of ghrelin on the cardiovascular system, and further demonstrated this in our GHSR-knockout mice model of atherosclerosis. However, these results are based on the mouse model and may still be limited in human atherosclerosis. In future studies, hybridizing different mouse types, such as ApoE^{-/-} and GHSR^{-/-} mice, may be necessary to further validate the role of ghrelin in atherosclerotic plaque formation and stabilization.

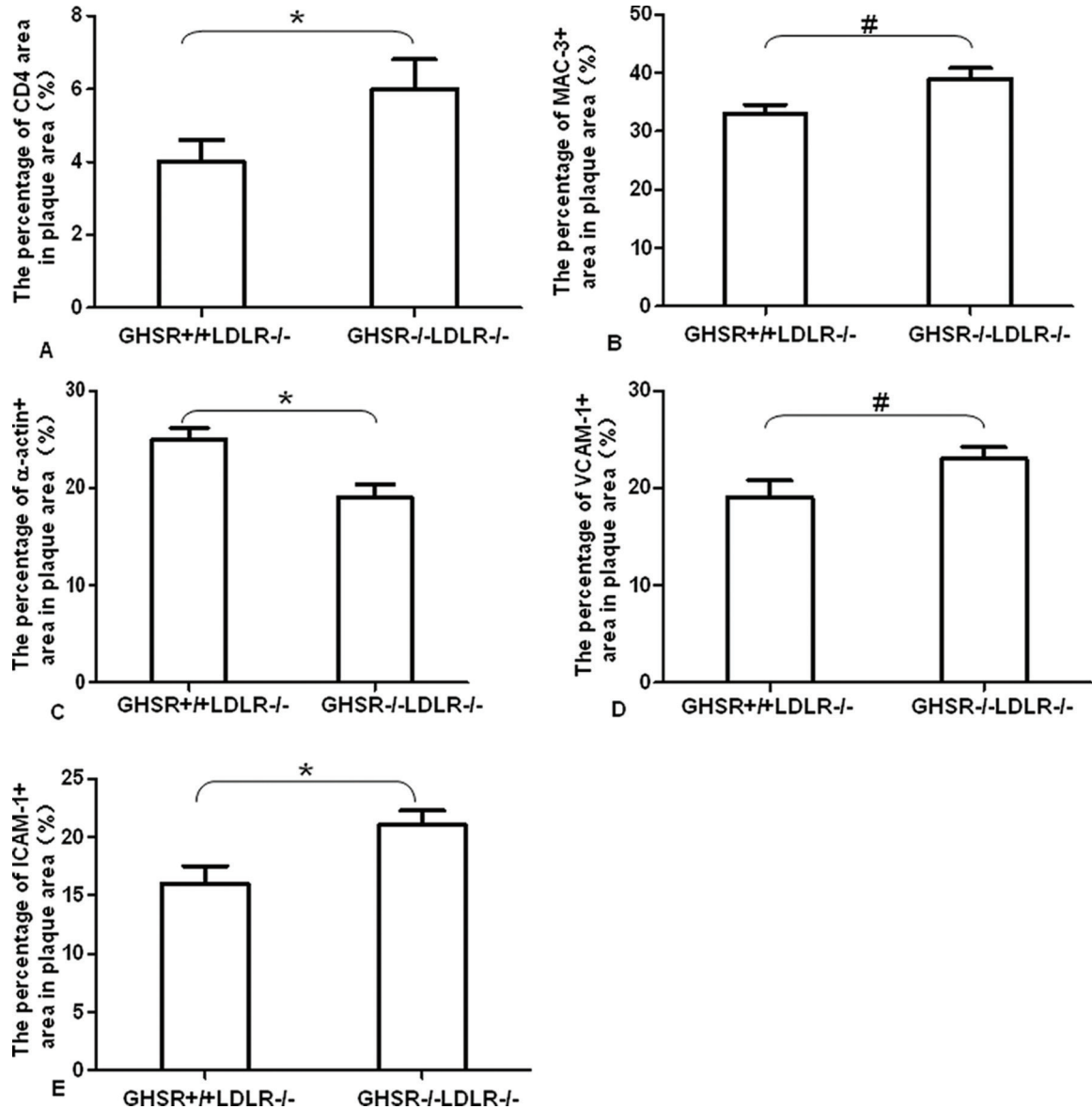


Figure 4. The percentage of CD4+ T, MAC-3+, α-actin+, VCAM-1+, and ICAM-1+ areas in plaque of GHSR^{-/-}LDLR^{-/-} and GHSR^{+/+}LDLR^{-/-} mice. A: CD4+ T area; B: MAC-3+ area; C: α-actin+ area; D: VCAM-1+ area; E: ICAM-1+ area. *: p<0.0.1; #: p<0.0.5.

6. CONCLUSION

In conclusion, lipid infiltration, and expression of CD4+ T cells, MAC-3+ macrophages, and adhesion molecules VCAM-1 and ICAM-1 in aortic arches are increased in GHSR^{-/-}LDLR^{-/-} mice, compared with GHSR^{+/+}LDLR^{-/-} mice. Thus, ghrelin receptor deficiency aggravates atherosclerotic plaque instability and vascular inflammation; this

information may provide novel avenues for the treatment of patients with atherosclerosis.

7. ACKNOWLEDGEMENTS

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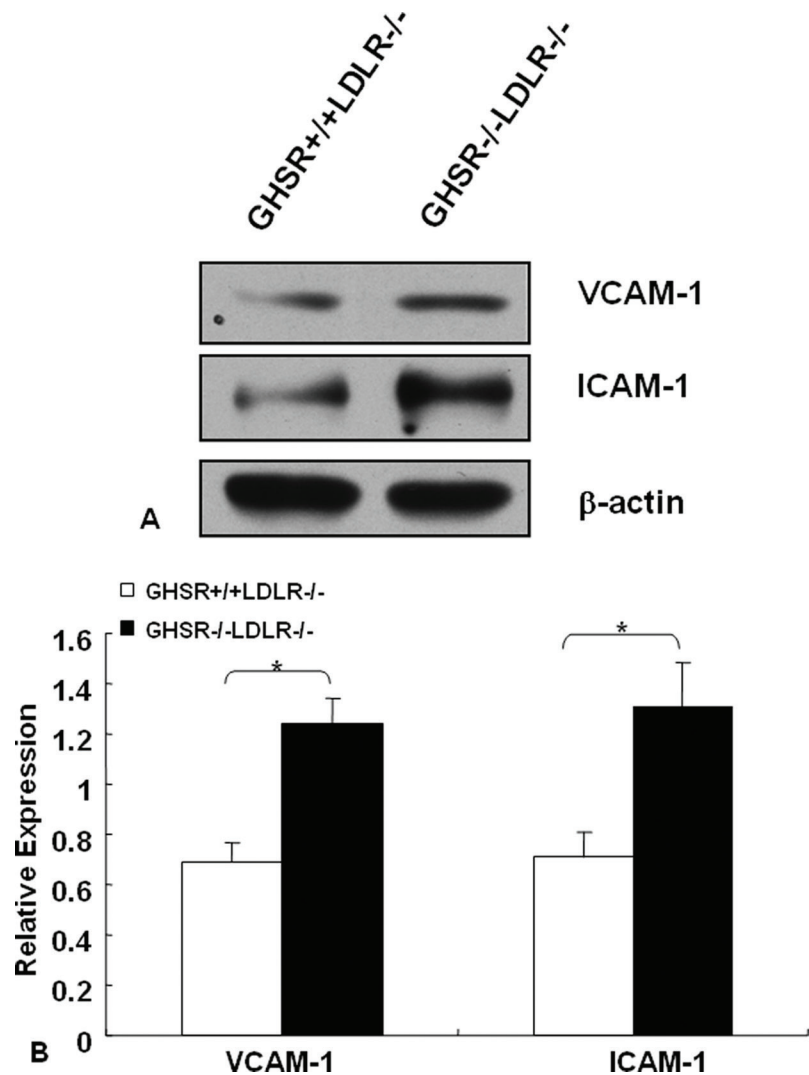


Figure 5. Western blotting (A) and quantitation of protein bands (B) showing differentially expressed VCAM-1 and ICAM-1 in the aortic arches of GSHR^{-/-}LDLR^{-/-} and GSHR^{+/+}LDLR^{-/-} mice. The results are representative of 12 GSHR^{-/-}LDLR^{-/-} mice and 12 GSHR^{+/+}LDLR^{-/-} mice. *: p<0.01.

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Abbreviations: GHSR: growth hormone secretagogue receptor; HUVECs, human umbilical vein endothelial cells; HDL: high-density lipoprotein; LDL: low-density lipoprotein; ICAM-1: intercellular cell adhesion molecule-1; PI3K: phosphoinositide 3-kinase

Key Words: Ghrelin, atherosclerotic plaque, ICAM-1, VCAM-1

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