Comparison of Apolipoprotein E Knockout Mice and Spontaneously Hyperlipidemic Mice in *Porphyromonas gingivalis*-Induced Atherosclerosis

日本大学松戸歯学部　口腔免疫学講座

研究講座員　　富田　直也

助教　　瀧澤　智美

准教授　　落合　智子

（指導：　渋谷　鑛教授）

(指導：　山本　正文教授)

Abstract

Porphyromonas gingivalis was shown to accelerate atheroma formation in a murine model such as apolipoprotein E knockout (ApoE-KO) mice and BALB/c. apoE-deficient spontaneously hyperlipidemic (C. KOR-Apoe^{shl}) mice. The purpose of this study is to characterize mouse gender and strain differences in *P. gingivalis*-induced atherosclerosis. Male and female ApoE-KO mice (10 weeks old) and age- and sex-matched C. KOR-Apoe^{shl} mice were intravenously injected with *P. gingivalis* three times a week for 3 weeks. Mice were then killed at 14 weeks. Atherosclerotic plaques in the proximal aorta were determined by Oil Red O staining. Serum lipid parameters (total cholesterol, TC; high density lipoprotein, HDL; and low density lipoprotein, LDL) and body weights were evaluated. *P. gingivalis* injection significantly enhanced the formation of atherosclerotic plaque in both ApoE-KO and Apoe^{shl} mice. Moreover, atherosclerotic lesion area was slightly larger in ApoE-KO mice than in Apoe^{shl} mice, while both model mice displayed similar body weight gain regardless of bacterial infection. The weight gain of male was larger than that of female in the period, and the TC of Apoe^{shl} mice was higher than that of ApoE-KO mice. Furthermore, *P. gingivalis* injection had elevated serum TC and LDL levels compared to the non-injected Apoe^{shl} mice group. These results suggest that *P. gingivalis* induces atherosclerosis in both ApoE-KO and Apoe^{shl} mice subsequently increasing TC and LDL levels. Thus, the Apoe^{shl} mice may sereve as a substitute for ApoE-KO mice at the point of it being more

natural and superior in examination of temporal change of morbidity, if we take account the import cost and the complication of maintenance of genetically modified mice.

Introduction

Atherosclerosis is a multifactorial cardiovascular disease (CVD), which is a leading cause of mortality and morbidity in western countries. Atherosclerosis is related to physiological and behavioral risk factors such as age, gender, hypertension, hypercholesterolemia, obesity, diabetes, smoking, and a sedentary lifestyle (1). Periodontitis also increases the risk of atherosclerosis, and previous studies have suggested that chronic infection with a periodontal pathogen, such as *Porphyromonas gingivalis*, is associated with an increased risk of atherosclerosis (2). Indeed, *P. gingivalis* was detected more frequently than other periodontal bacteria in atherosclerotic plaques (3).

P. gingivalis promotes platelet aggregation, increases systemic inflammatory markers, invades endothelium and vascular smooth muscle cells, and alters endothelial function (2). Furthermore, *P. gingivalis* accelerates the progression of atherosclerosis in homo- and heterozygous apolipoprotein E (ApoE)-deficient mice, rabbits, and pigs (2). Since ApoE knockout (KO) mice form remarkable atheromatous plaques under normal chow diet feeding, they are famous as model of arteriosclerosis (4, 5). ApoE is a main component of plasma lipoproteins synthesized by liver and has a very high affinity for the Low-Density Lipoprotein (LDL) receptor in accordance with ApoB-100. ApoE is mainly promoting the clearance to liver out of the blood of an ApoB-100 content lipoproteins such as Very Low Density Lipoprotein (VLDL) and Intermediate Density Lipoproteins (IDL). Many arteriosclerosis researches are done using the ApoE-KO mice

(6).

On the other hand, ApoE-deficient, spontaneously hyperlipidemic (KOR-Apoe^{shl}) mice, which is an inbred strain created from Japanese wild mice, are also deficient in the expression of ApoE because of a gross disruption in the *Apoe* gene (7). These mice are hypercholestrolemic and accumulate large amounts of remnant-like particles in the bloodstream, as has been observed in ApoE-KO mice. Several congenic ApoE-deficient mice with the genetic background of laboratory mice were developed by transferring the *Apoe* gene mutation from a KOR genetic background (8). An alternative ApoE-deficient murine model may be useful given the complexities involved in importing and maintaining genetically modified animals. However, in two groups of mice whose *Apoe* gene was disrupted by homologous recombination or by naturally occurring mutation, their weight change, lipid composition and atherosclerosis formation are not compared until now. Therefore, we compared KOR-Apoe^{shl} mice having a BALB/c background (c.KOR-Apoe^{shl}) with ApoE-KO mice about weight change, the lipid composition of a lipoprotein, and the degree of *P. gingivalis*-induced atherosclerosis.

Materials and methods

Bacterial strain

P. gingivalis strain 381 was cultured on anaerobic blood agar plates (Becton Dickinson, Sunnyvale, CA) in a model 1024 anaerobic system (Forma Scientific, Marietta, OH) with 10% H_2, 80% N_2, and 10% CO_2 for 3–5 days. Cultures were then inoculated into brain heart infusion broth (Difco, Detroit, MI), supplemented with 5 μg of hemin/ml and 0.4 μg of menadione/ml, and grown for 2 days, reaching an optical density at 660 nm (OD_{660}) of 0.8, corresponding to 10^9 CFU/ml (9). The cultured cells were then centrifuged at 8000 × *g* for 20 min at 4°C and diluted with phosphate-buffered saline (PBS) for intravenous (i.v.) infection.

Mice and treatments

Ten-week-old male and female ApoE-KO mice obtained from Jackson Laboratories (Bar Harbor, ME) or age- and sex-matched c.KOR-Apoe^{shl} mice obtained from Japan SLC. Inc, were divided randomly into two groups (n = 6 for each group). The institutional Animal Care and Use Committee of Nihon University approved all animal protocols (Approval number AP12MD022). The mice were given regular mouse chow and water ad libitum. The mice were inoculated intravenously three times a week for 3 weeks with 0.1 ml of live *P. gingivalis* (10^8 CFU /mouse) or 0.1 ml of PBS. All mice were monitored daily until death. At the age of 14 weeks, the mice from each group were killed, and tissue and blood samples were collected.

Quantification of the atherosclerotic lesion area

Blood was collected into heparinized syringes from the orbital veins of mice anesthetized with Isozol (Nichi Iko, Toyama, Japan). The heart and aortic tree were then perfused through the left ventricle with ice-cold 0.9% PBS for 10 min. The heart was then carefully dissected and removed. The upper half of the heart containing the aortic origin was separated and embedded in Tissue-Tek OCT compound (Fisher Scientific, Newark, DE) in cryomolds, and cryostat sections were prepared (10). Using a modified version of the method of Paigen *et al.* (10), cryosections of the aortic arch for atherosclerotic plaque accumulation by Oil Red O staining were examined. The lesion area was then quantified under a microscope (BX51; Olympus, Tokyo, Japan), interfaced with a CCD camera and an image analysis system (Lumina Vision; Mitani Co., Fukui, Japan). Briefly, cross-sectional areas from three images were summed to obtain the total lesion area per slide, and the percentage of the aortic lumen occupied by lesions per section was calculated. Slides were analyzed in a blinded manner, and the percentage of the aortic lumen occupied by lesions was averaged over 15 sections per animal, expressed as the percentage of the lumen of the proximal aorta occupied by lesions per section, per animal.

Measurement of serum lipids

Serum was isolated from the blood by centrifugation at 2500 x *g* for 20 min after clotting at room temperature. The serum level of total cholesterol (TC), LDL, or

high-density lipoprotein (HDL) was determined using a quantitative kit (Wako Pure Chemicals, Tokyo, Japan).

Statistical analysis

Results are expressed as the mean ± the standard errors (SE). Data were analyzed using the 2-tailed Student's test or one-way ANOVA, followed by Tukey–Kramer multiple tests. $P < 0.05$ was considered statistically significant.

Results

Body weight

We compared the body weights of Apoe^{shl} mice and ApoE-KO mice at 10 to 14-weeks of age (Fig. 1, n = 6). Both groups of mice had a similar weight gain at 14 weeks (Fig. 1A and B, 29.9 ± 1.4 g for Apoe^{shl} male mice vs. 29.0 ± 1.0 g for ApoE-KO male mice; 25.7 ± 0.8 g for Apoe^{shl} female mice vs. 26.7 ± 1.2 g for ApoE-KO female mice). In any of the mouse strains, the weight gain of male in the period was larger than that of female (Fig. 1A and B, 29.9 ± 1.4 g for Apoe^{shl} male mice vs. 25.7 ± 0.8 for Apoe^{shl} female mice, $P < 0.05$; 29.0 ± 1.0 g for ApoE-KO male mice vs. 26.7 ± 0.8 g for ApoE- KO female mice, $P < 0.05$). Furthermore, *P.gingivalis* challenge slightly increased body weight of the Apoe^{shl} female mice and the ApoE-KO male mice (Fig. 1A and B, 25.7 ± 1.8 g for Apoe^{shl} female mice vs. 26.7 ± 1.3 for Apoe^{shl} female mice challenged with *P. gingivalis*; 29.0 ± 1.6 g for ApoE-KO male mice vs. 30.1 ± 1.9 g for ApoE-KO male mice challenged with *P. gingivalis*).

Histomorphometric analysis of the aortic sinus

We examined cryosection of the aortic sinus for atherosclerotic plaque accumulation by Oil Red O staining. Histomorphological analysis revealed an increase in atherosclerotic plaque accumulation in *P. gingivalis*-challenged Apoe^{shl} mice compared to sham-treated Apoe^{shl} mice, with the percentage of the total lumen of the proximal aorta occupied by lesions showing the same pattern (Fig. 2A and B, 1.7 ± 0.3 % vs.0.3 ±

0.1% for male, $P < 0.05$; 1.4 ± 0.3% vs. 0.2 ± 0.1% for female, $P < 0.05$). Although *P. gingivalis*–challenged male mice formed the slightly larger arteriosclerosis focus than female mice, the statistical significant difference was not accepted among both groups. Similar results were also obtained in ApoE-KO mice. *P. gingivalis* challenge resulted in significant increase from 0.1 ± 0.04 to 2.8 ± 0.2 for male (Fig. 3A and B, $P < 0.05$) and from 0.5 ± 0.3 to 2.4 ± 0.5 for female (Fig. 3A and B, $P < 0.05$) in ApoE-KO mice. These also showed the same tendency that *P. gingivalis*–challenged male mice formed the slightly larger arteriosclerosis lesions than female mice, however the statistical significant difference was not accepted among both groups.

Serum cholestrol level

TC and LDL levels in Apoe^{shl} and ApoE-KO male mice were higher than those of female mice regardless of the existence of infection (Fig. 4A and B). TC and LDL levels in Apoe^{shl} male mice were increased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4A; TC, 807 ± 14 vs. 701 ± 11 mg/dL, $P<0.05$; LDL, 310 ± 7 vs. 210 ± 8 mg/dL, $P<0.05$). TC and LDL levels in Apoe^{shl} female mice were also increased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4A; TC, 700 ± 7 vs. 578 ± 7 mg/dL, $P<0.05$; LDL, 196 ± 5 vs. 166 ± 4 mg/dL). On the other hand, HDL levels in Apoe^{shl} male and female mice were decreased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4A; HDL, 35 ± 2 vs. 40 ± 2 mg/dL for male; 32 ± 1 vs. 42 ± 1 mg/dL for female). Furthermore, even in ApoE-KO mice, the similar tendency was observed (Fig. 4B). TC and LDL

levels in ApoE-KO male mice were increased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4B; TC, 636 ± 26 vs. 600 ± 13 mg/dL; LDL, 229 ± 21 vs. 200 ± 17 mg/dL). TC and LDL levels in ApoE- KO female mice were also increased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4B; TC, 512 ± 21 vs. 455 ± 32 mg/dL; LDL, 193 ± 17 vs. 175 ± 10 mg/dL). On the other hand, HDL levels in ApoE-KO male and female mice were decreased in *P. gingivalis*-challenged mice when compared to sham-treated mice (Fig. 4A; HDL, 58 ± 4 vs. 62 ± 4 mg/dL for male; 32 ± 2 vs. 49 ± 3 mg/dL for female).

Discussion

A growing body of evidence indicates a clear relationship between hypercholesterolemia and atherosclerosis in humans (11-13). However, similar levels of hyperlipidemia in different individuals are not necessary result in similar levels of atherosclerosis in these individuals. This individuality is probably due to many factors including associated diseases (e.g. diabetes mellitus), life style, age and genetic background. Since family histories often exist in person with atherosclerosis, genetic backgrounds would certainly be a risk factor. Therefore, in this study, we examined the distinction in severity of atherosclerosis between two lines of ApoE-deficient mice, i.e., KOR-Apoe^{shl} and ApoE-KO mice, challenged with periodontopathic bacteria.

The present study resulted that *P. gingivalis* challenge significantly increased atherosclerosis plaque accumulation in both of ApoE-KO and Apoe^{shl} mice, although atherosclerotic lesion area was slightly larger in ApoE-KO mice than Apoe^{shl} mice. About gender difference, any ApoE-deficient male mice formed the bigger atherosclerosis focus compared with the age matched females. Indeed, men are more likely to die of CVD than women. Population studies demonstrate a male to female ratio in coronary artery disease (CAD) mortality ranging from 2.5 to 1 to 4.5 to 1 in countries with different rates of CAD (14). As a reason for that, our results indicated that male mice had a greater propensity of gaining body weight than female mice in any of Apoe^{shl} and ApoE-KO mice. These results suggest that male mice are more likely to become obese than female mice. Obesity is also a risk factor for adult coronary heart

disease and is increasing in prevalence among youths as well as adults (15). Longitudinal data supports obesity is associated with increased mortality due to CVD (16). Hypercholesterolemia is frequently found in patients with obesity, so that the average serum cholesterol level is significantly higher in overweight subjects than in lean ones, and usually a significant correlation exists between serum cholesterol and obesity (17, 18). Indeed, male mice had higher serum cholesterol and LDL cholesterol concentrations compared to female mice in any of $Apoe^{shl}$ mice and ApoE-KO mice.

In this study, *P. gingivalis* challenge promoted arteriosclerosis with weight gain and the increase in TC and LDL. An increased level of LDL is a very well established risk factor of CAD. Previous study had also shown that the systemic inflammation induced by oral infection with *P. gingivalis* is associated with a shift to a proatherogenic serum lipid profile that leads to elevated LDL and decreased HDL levels (19). Plasma cholesterol level is regulated by the LDL receptor and the expression of the LDL receptor is regulated at transcriptional and post transcriptional levels.

Recently, stimulation with lipopolysaccharide of *P. gingivalis* induced the change of cholesterol transport via targeting the expression of LDL receptor-related genes and resulted in the disturbance of regulatory mechanisms of the cholesterol level in macrophages (20). Therefore, *P. gingivalis* challenge may accelerates atherosclerosis lesion formation by altering the lipid profile. In patients with periodontitis, total cholesterol and LDL cholesterol levels were also increased and HDL cholesterol levels were decreased compared to those in healthy subjects (21-23).

In conclusion, our findings suggest that differences between males and females in their response to *P. gingivalis* challenge should be explored in representative murine models in atherosclerosis. Although the different associations between body weights and serum lipid profile such as TC, LDL, or HDL, were observed in two lines ApoE-deficient male and female mice, *P. gingivalis* challenge accelerated atheroma lesion formation in both of mice strains regardless of gender. Therefore, $Apoe^{shl}$ mice may be more natural and more useful in the understanding of change over time of morbidity, if we take into account the import cost and the complication of maintenance of genetically modified mice.

References

1. Stocker R, Keaney JF, Jr: Role of oxidative modifications in atherosclerosis. Physiological reviews, 84: 1381-1478, 2004.
2. Hayashi C, Gudino CV, Gibson FC, 3rd, Genco CA: Review: Pathogen-induced inflammation at sites distant from oral infection: bacterial persistence and induction of cell-specific innate immune inflammatory pathways. Mol Oral Microbial, 25: 305-316, 2010.
3. Pucar A, Milasin J, Lekovic V, Vukadinovic M, Ristic M, Putnik S, Kenney EB: Correlation between atherosclerosis and periodontal putative pathogenic bacterial infections in coronary and internal mammary arteries. J Periodontol, 78: 677-682, 2007.
4. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL: Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. Cell, 71: 343-353, 1992.
5. Meir KS, Leitersdorf E: Atherosclerosis in the apolipoprotein-E-deficient mouse: a decade of progress. Arterioscler Thromb Vasc Biol, 24: 1006-1014, 2004.
6. Jawien T. The role of an experimental model of atherosclerosis: apoE-knockout mice in developing new drugs against atherogenesis. Curr Pharm Biotechnol, 13: 2435-2439, 2012.
7. Matsushima Y, Hayashi S, Tachibana M: Spontaneously hyperlipidemic (SHL) mice:

Japanese wild mice with apolipoprotein E deficiency. Mamm Genome, 10: 352-357, 1999.

8. Matsushima Y, Sakurai T, Ohoka A, Ohnuki T, Tada N, Asoh Y, Tachibana M: Four strains of spontaneously hyperlipidemic (SHL) mice: Phenotypic distinctions determined by genetic backgrounds. J Atherosclerosis Thromb, 8: 71-79, 2001.
9. Koizumi Y, Kurita-Ochiai T, Oguchi S, Yamamoto M. Nasal immunization with *Porphyromonas gingivalis* outer membrane protein decreases *P. gingivalis*-induced atherosclerosis and inflammation in spontaneously hyperlipidemic mice. Infect Immun, 76: 2958-2965, 2008.
10. Paigen B, Morrow A, Holmes PA, Mitchell D, Williams RA: Quantitative assessment of atherosclerotic lesions in mice. Atherosclerosis, 68: 231-240, 1987.
11. Sloop GD: A critical analysis of the role of cholesterol in atherogenesis. Atherosclerosis, 142: 265-268, 1999.
12. Henry RY, Kendall MJ: Does cholesterol lowering prevent stroke? J Clin. Pharm. Ther, 23: 337-344, 1998.
13. Patel ST, Kent KC: Risk factors and their role in the diseases of the arterial wall. Semin Vasc Surg, 11: 156-168, 1998.
14. Kalin MF, Zumoff B: Sex hormones and coronary disease: a review of the clinical studies. Steroids, 55: 330-52, 1990.
15. Miettinen TA: Cholesterol production in obesity. Circulation, 44:842-850, 1971.
16. Jiang J, Ahn J, Huang WY, Hayes RB: Association of obesity with cardiovascular disease mortality in the PLCO trial. Prev Med, 57: 60-64, 2013.

17. Tanner JM: The ralation between serum cholesterol and physique in healthy young men. J Physiol, 115: 371-390, 1951.

18. Montoye HJ, Epstein FH, Kjelsberg MO: Relationship between serum cholesterol and body fatness. An Epidemiologic Study. Am J Clin Nutr, 18: 397-406, 1966.

19. Khovidhunkit W, Kim MS, Memon RA, Shigenaga JK, Moser AH. Feingold KR, Grunfeld C: Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. J Lipid Res, 45: 1169-1196, 2004.

20. Maekawa T, Takahashi N, Tabeta K, Aoki Y, Miyashita H, Miyauchi S, Miyazawa H, Nakajima T, Yamazaki K: Chronic oral infection with *Porphyromonas gingivalis* accelerates atheroma formation by shifting the lipid profile. PLoS One, 6: e20240, 2011.

21. D'Aiuto F, Parkar M, Nibali L, Suvan J, Lessem J. Tonetti MS: Periodontal infections cause changes in traditional and novel cardiovascular risk factors: results from a randomized controlled clinical trial. Am Heart J, 151: 977-984, 2006.

22. Ramirez-Tortosa MC, Quiles JL, Battino M, Grandos S, Morillo JM, Bopadre S, Newman HN, Bullon P: Periodontitis is associated with altered plasma fatty acids and cardiovascular risk markers. Nutr Metab Cardiovasc Dis, 20: 133-139, 2010.

23. Rufail ML, Schenkein HA, Koertge TE, Best AM, Barhour SE, Tew JG, van Antwerpen R: Atherogenic lipoprotein parameters in patients with aggressive periodontitis. J Pediodont Res, 42: 495-502, 2007.

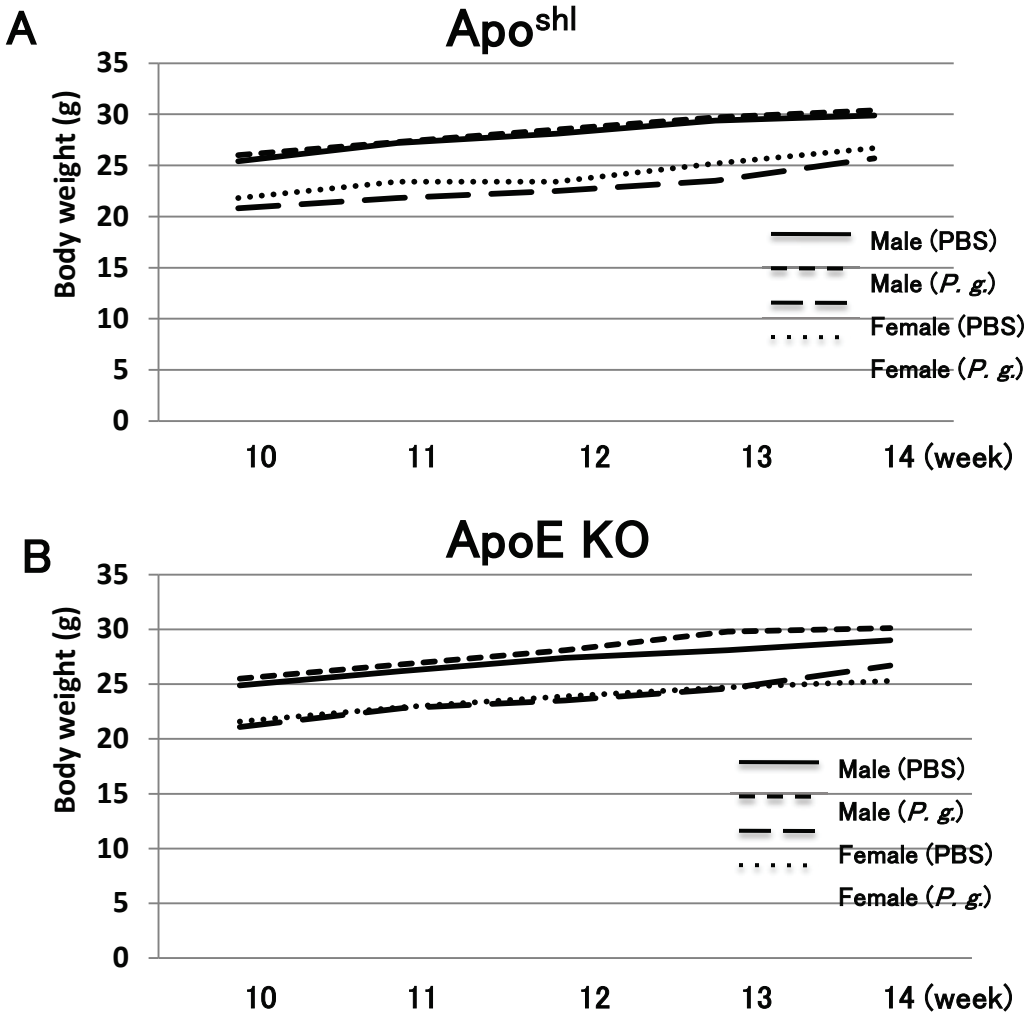

Fig. 1. Body weight of male and female in Apoe^{shl} (A) and ApoE-KO (B) mice. Mice were divided into four groups for each strain. Each mouse was weighted twice weekly until sacrificed.

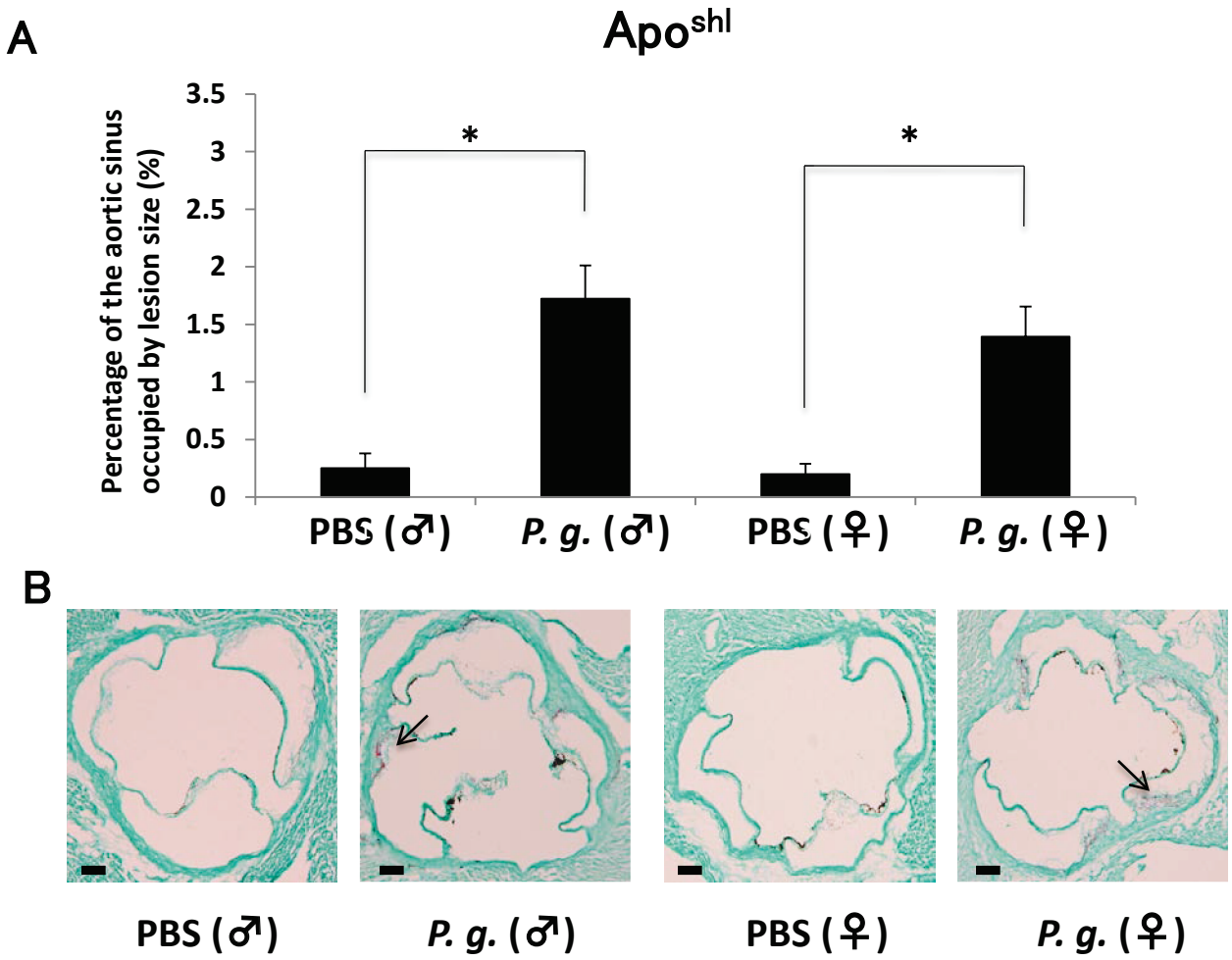

Fig. 2. Atherosclerotic plaque formation in the aortic sinuses of Apoe^{shl} mice intravenously injected with *P. gingivalis*. The result of a histomorphometric analysis of the percentage of the aortic sinus occupied by lesions (A), is shown at 15 weeks. The data represent the mean ± SE (n = 6). *P < 0.05 compared to the PBS-inoculated group. (B) Oil Red O-stained cryosections of the proximal aorta. Arrows indicate typical lipid-rich atherosclerosis areas stained with Oil Red O. Scale bars = 50 μm.

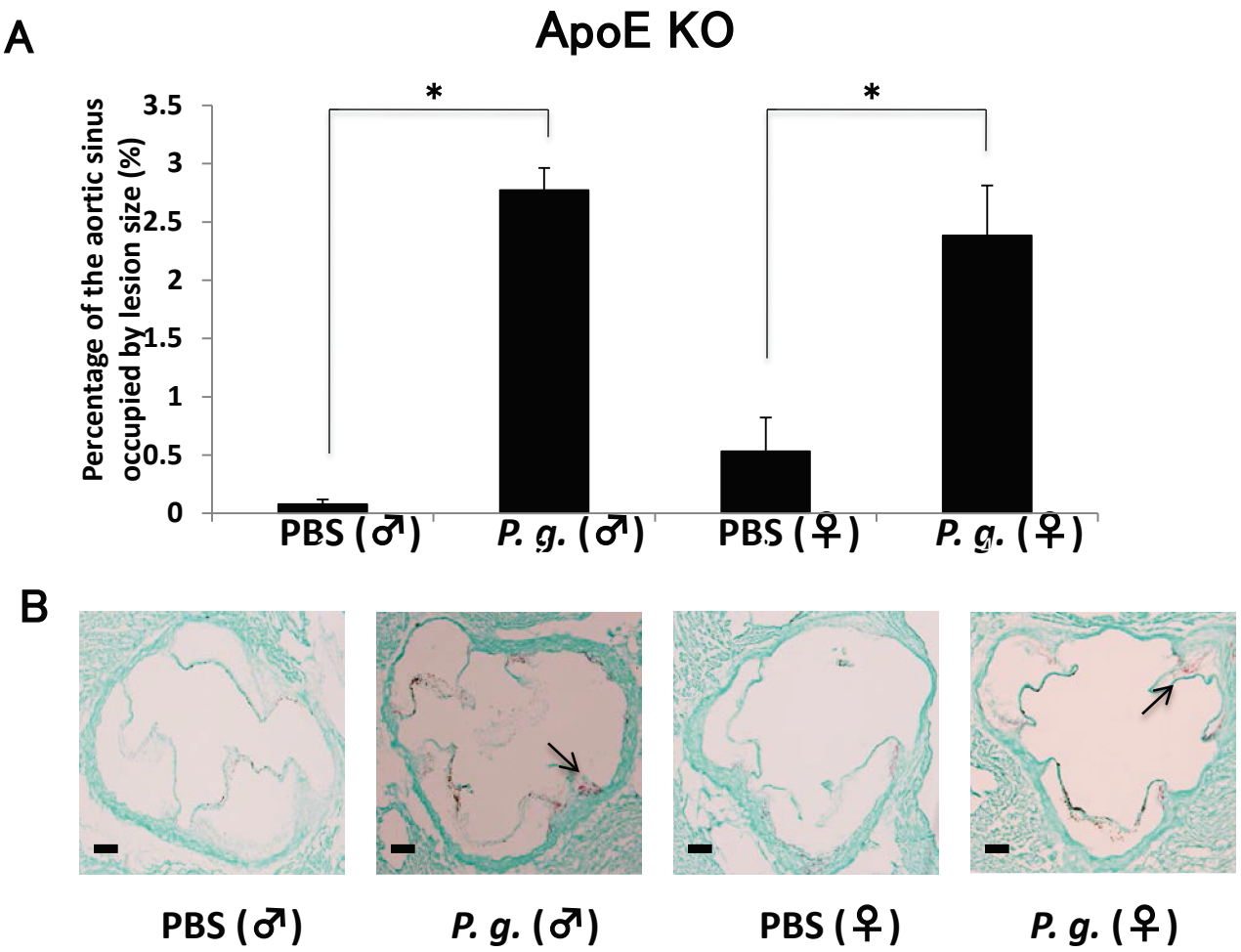

Fig. 3. Atherosclerotic plaque formation in the aortic sinuses of ApoE-KO mice intravenously injected with *P. gingivalis*. The result of a histomorphometric analysis of the percentage of the aortic sinus occupied by lesions (A), is shown at 15 weeks. The data represent the mean ± SE ($n = 6$). *$P < 0.05$ compared to the PBS-inoculated group. (B) Oil Red O-stained cryosections of the proximal aorta. Arrows indicate typical lipid-rich atherosclerosis areas stained with Oil Red O. Scale bars = 50 μm.

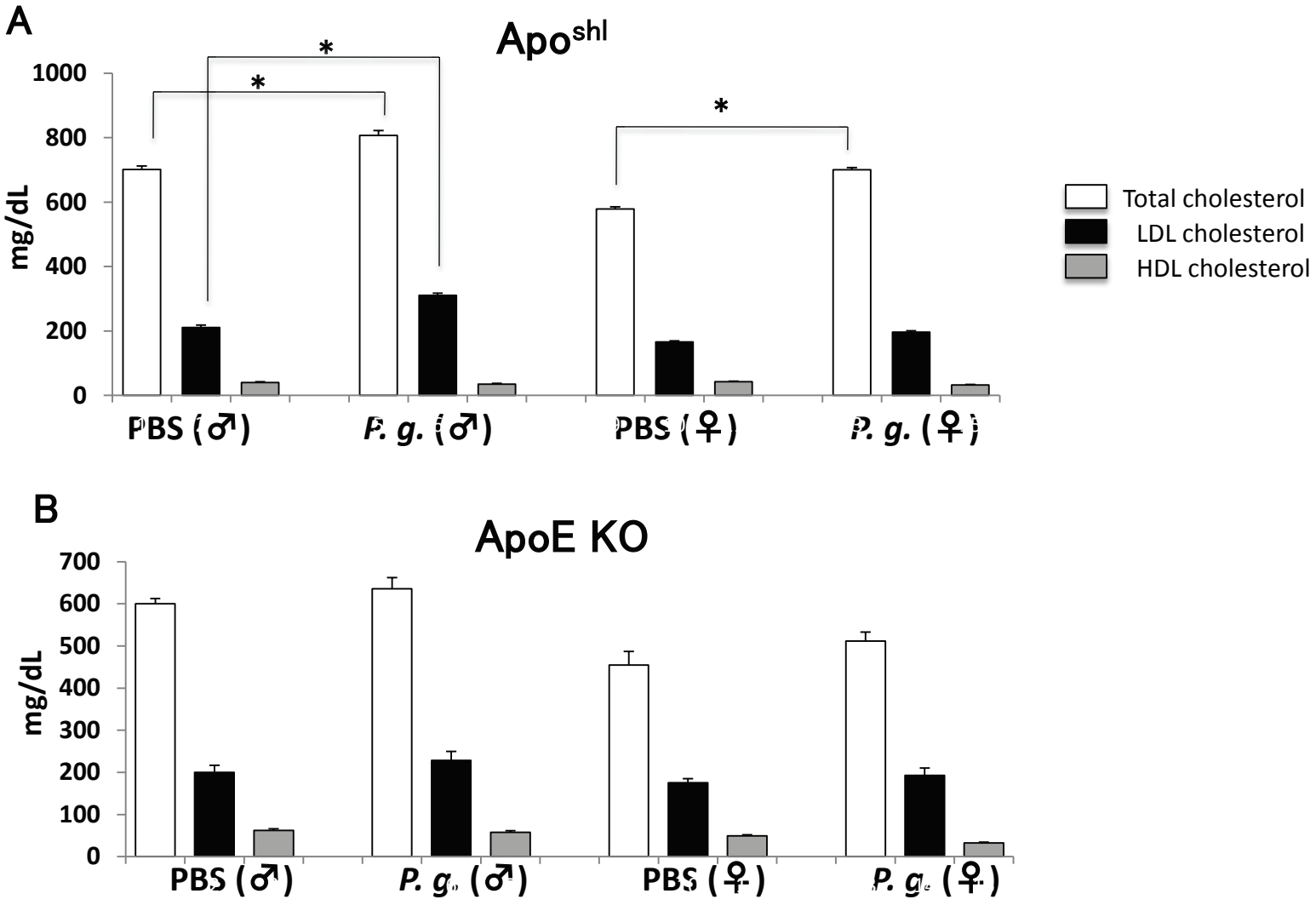

Fig. 4. TC, LDL, and HDL levels in Apoe^{shl} (A) and ApoE-KO (B) mice. Black, white, and grey bars represent TC, LDL, and HDL, respectively. The data represent the mean ± SE ($n = 6$). *$P < 0.05$ compared to the PBS-inoculated group.