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INCREASED AORTIC ATHEROSCLEROTIC PLAQUE DEVELOPMENT IN FEMALE APOLIPOPROTEIN E-NULL MICE IS ASSOCIATED WITH ELEVATED THROMBOXANE A_2 AND DECREASED PROSTACYCLIN PRODUCTION

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The production of thromboxane A_2 (TXA_2) and prostacyclin (prostaglandin I_2, PGI_2) is known to be increased in patients with atherosclerosis. In this study, we evaluated the influence of gender on TXA_2 and PGI_2 production, and their association with the progression of atherosclerosis in apolipoprotein E-null ($ApoE^{-/-}$) mice maintained on a high fat diet for 3 months. En face analyses of aortas showed marked increases in plaque formation in female $ApoE^{-/-}$ mice. Quantification of the hematoxylin/eosin (H&E) stained cross sections of the aortic arch revealed 3 to 4-fold higher plaque thickness in female $ApoE^{-/-}$ mice. Analyses of 24-hours urine samples for 11-dehydro TXB_2 and 2, 3-dinor-6-keto $PGF_{1\alpha}$ indicated that female $ApoE^{-/-}$ mice produce up to 15-fold more TXA_2 and 50% less PGI_2 than the age matched males. Interestingly, the serum cholesterol levels in $ApoE^{-/-}$ females were 20% lower than males on the high fat regimen. No gender-associated changes in the number of T lymphocytes, mast cells and macrophages were evident in the lesion areas of $ApoE^{-/-}$ mice. The results suggest that the markedly elevated TXA_2 production and reduced PGI_2 production are gender-related proatherogenic risk factors in female $ApoE^{-/-}$ mice.

Key words: *$ApoE^{-/-}$ mice, atherosclerosis, gender difference, thromboxane A_2, prostaglandin I_2, estrogen, high fat diet*

INTRODUCTION

Atherosclerotic cardiovascular disease causes the highest incidence of morbidity and mortality in the affluent nations of the world. Atherosclerosis is a multifactorial disease and its pathogenesis involves a number of inflammatory responses including endothelial cell activation, enhanced expression of adhesion molecules and inflammatory cytokines, leukocyte, macrophage and mast cell accumulation, and alterations in the expression of cyclooxygenases and prostanoid synthases (1-4). Although atherosclerosis is more prevalent in the elderly, irrespective of the race or ethnicity, men appear to be more prone to develop the disease than women during their reproductive years. Furthermore, the finding that post-menopausal women are at an equal risk as men of comparable age, led to the hypothesis that estrogen might be acting as a cardio-protective agent in pre-menopausal women. Opposing views to this hypothesis are also evident from the reports which show Tamoxifen, an estrogen receptor antagonist, in some tissues, acts as a cardioprotective agent in several mouse models of atherosclerosis (5, 6), and in postmenopausal women who used it as an adjuvant therapy for breast cancer (7-10). These findings raise the questions regarding the cardioprotective effects of estrogen in women (11).

Thromboxane A_2 (TXA_2) and prostacyclin (prostaglandin I_2, PGI_2) are products of the cyclooxygenase (COX) pathway and have earned considerable interest in cardiovascular physiology because of their distinct effects on vascular functions (12, 13). PGI_2 is a major prostanoid generated by endothelial cells, and is a potent vasodilator and an inhibitor of leukocyte adhesion and platelet aggregation. Therefore, PGI_2 is considered to be both anti-atherothrombotic and cardioprotective. On the other hand, TXA_2 is a potent inducer of vasoconstriction, platelet activation, and platelet adhesion, and is a proatherogenic prostanoid. Since TXA_2 and PGI_2 exert opposing effects in the vasculature, their relative concentrations in the circulation and microenvironment are critical for the normal cardiovascular function and prevention of atherosclerotic progression. The objectives of the present study were to determine the influence of gender on TXA_2 and PGI_2 production and their relationship to the progression of atherosclerosis in the apolipoprotein-E gene knockout ($ApoE^{-/-}$) mouse model. The results presented in this report demonstrate that female $ApoE^{-/-}$ mice develop markedly increased aortic atherosclerotic lesions and intima/media thickness compared to males. The increased susceptibility of female $ApoE^{-/-}$ mice to atherosclerotic progression is associated with marked elevation in the systemic production of TXA_2 and decreased PGI_2 production with a consequent shift in the TXA_2/PGI_2 ratio in favor of atherogenesis.

MATERIALS AND METHODS

Animals, diets, and specimen collections

Eight to ten week-old ApoE$^{-/-}$ mice of both genders and their C57Bl/6 wild type (WT) controls were obtained from the Jackson Laboratories (Bar Harbor, Maine). They were fed *ad libitum* for 3 months with a high fat diet (Western Diet, TD.88137, Harlan Teklad, Madison, WI) containing 17.3% protein, 48.5% carbohydrate, 21.2% fat, and 0.2% cholesterol by weight, and 42% kcal from fat. At the end of the regimen, 24-hour urine samples were collected by placing each mouse in a metabolic cage (Tecniplast, Rochester, NY). Blood samples were collected by bleeding from the retro-orbital sinus under anesthesia, or at the time of necropsy. Mice were euthanized using isoflurane (Abbott, North Chicago, IL) inhalation, bled by snipping the hepatic vein, and the aorta was perfused *in situ* with PBS and buffered 10% formalin. All animal experiments were approved by the University of Kansas Medical Center Institutional Animal Care and Use Committee and performed in accordance with the protocol guidelines.

Assessment of atherosclerotic plaques in the aorta

After *in situ* perfusion with cold PBS and subsequently with cold buffered formalin, the arch and thoracic portion of the dorsal aorta were dissected free from the thoracic cavity and heart. The entire aorta was isolated from the arch to the aortic-iliac bifurcation. After removing the adventitia and adipose tissue it was placed in 10% neutral buffered formalin overnight. The aorta was then opened lengthwise, and pinned flat in a wax bottomed dissecting pan. The tissue was stained for 15 minutes with 0.5% Sudan IV solution (14) in acetone and 70% ethanol (1:1). The tissue was decolorized for 5 minutes using 80% ethanol, and then washed gently with water for several minutes. The en face preparations were digitally photographed and then quantified using Optimas 6.5 software, and percent of plaque coverage was calculated.

Histological evaluation of specimens

Sections of the arch and thoracic portions of the aorta were cut and stained with hemotoxylin/eosin (H&E) or Giesma stains. Histological sections were examined by light microscopy to evaluate the overall architecture with careful attention to atherosclerotic changes in the root, aortic arch, and the thoracic aorta, in addition to any other histopathology alterations. Tissue sections on glass slides were scanned using the Aperio Scanscope System (Vista, CA) to create virtual slides that were stored on the University of Kansas Medical Center server for histological evaluation. All virtual slides were analyzed at the same magnification, to accurately evaluate plaque thicknesses by the ruler tool. The thickest area of plaque was measured on sections of the root of the aorta. Measurements of intima thickness were normalized by the media thickness, and the mean ratio was used to determine differences between groups.

Measurement of thromboxane and prostacyclin products in the urine

The levels of 11-dehydro thromboxane B_2 and 2, 3-dinor-6-keto prostaglandin $F_{1\alpha}$in the 24-hour urine samples were quantified by using competitive EIA kits to evaluate the systemic production of TXA_2 and PGI_2, respectively. The values for the prostanoid metabolites in the 24-h urine samples were normalized for the creatinine content. The measurement of 2, 3-dinor-6-keto prostaglandin $F_{1\alpha}$ required purification and spiking protocols to obtain the final results. The creatinine assay kit (No. 500701) and EIA kits for 11-dehydro thromboxane B_2 (No. 519501) and 2, 3-dinor-6-keto prostaglandin $F_{1\alpha}$ (No. 515121), were purchased from Cayman Chemical (Ann Arbor, MI). The patterns of increased 11-dehydro thromboxane B_2 in female urine were further validated by gas chromatography/mass spectrometry.

Serum chemistry, lipid profiles, testosterone and estradiol

Serum chemistry and lipid profile were analyzed at the Veterinary Laboratory Resources, Shawnee Mission, KS, and the estradiol and testosterone levels in the serum samples were analyzed at the University of Virginia Center for Research in Reproduction, Ligand Assay and Analysis Core Laboratory (Charlottesville, VA).

Immunohistochemical detection and quantification of T cells, mast cells and macrophages

The presence of T cells, mast cells and macrophages were evaluated after immunostaining the paraffin sections with anti-CD3 (N1580, DAKO, Carpinteria, CA), anti-CD117 (14-1172, eBioscience, San Diego, CA), and anti-Mac-3 (14-5989, eBioscience), respectively. Hematoxylin was used as a counter stain. After completion of immunohistochemical staining, slides were placed on the ACIS™ automated imaging system (DAKO, Carpinteria, CA) for quantifying the tissue staining. The system consists of an automated microscope, a 3-chip Sony progressive scan camera, a computer, and Windows NT 4.0 workstation software interface. Each slide was scanned by the robotic microscope. The ACIS™ system captures images from each slide, quantifies staining, and presents a numerical score. It was used to quantify immunohistochemical staining intensity for Mac-3 and percent positive cells for CD3. An average score for all selected areas was then calculated for each marker. Mast cells were identified on slides stained with Giemsa and CD117 antibodies, and quantified by averaging the number of mast cells counted in five high power fields.

RESULTS

Female ApoE$^{-/-}$ mice develop larger atherosclerotic lesions than males

In order to compare the effects of gender on the extent of atherosclerotic lesions at 3 months, en face analyses of Sudan IV-stained thoracic aortic areas were quantified by digital imaging. As shown in *Fig. 1A*, female Apo-E$^{-/-}$ mice have marked increases in atherosclerotic lesions compared to males. Representative en face images of aortas from ApoE$^{-/-}$ mice depicting atherosclerotic lesions are presented in *Fig. 1B*.

The analyses of the H&E-stained cross sections of the aortic arch form ApoE$^{-/-}$ mice maintained on the high fat diet for 3 months revealed increased intima to media ratios in females when compared to males (*Fig. 2A*). Representative images of the cross sections of the root of the aortas depicting lesions from these mice are presented in *Fig. 2B*. Atherosclerotic lesions were not found in aortas of wild type controls of either gender after a 3-month high fat diet (data not shown).

Female ApoE$^{-/-}$ mice produce higher levels of thromboxane

Previous studies have shown that $TXA_2$and PGI_2 act in an opposing fashion in the initiation and progression of atherogenesis in ApoE$^{-/-}$ mice (15). In order to determine

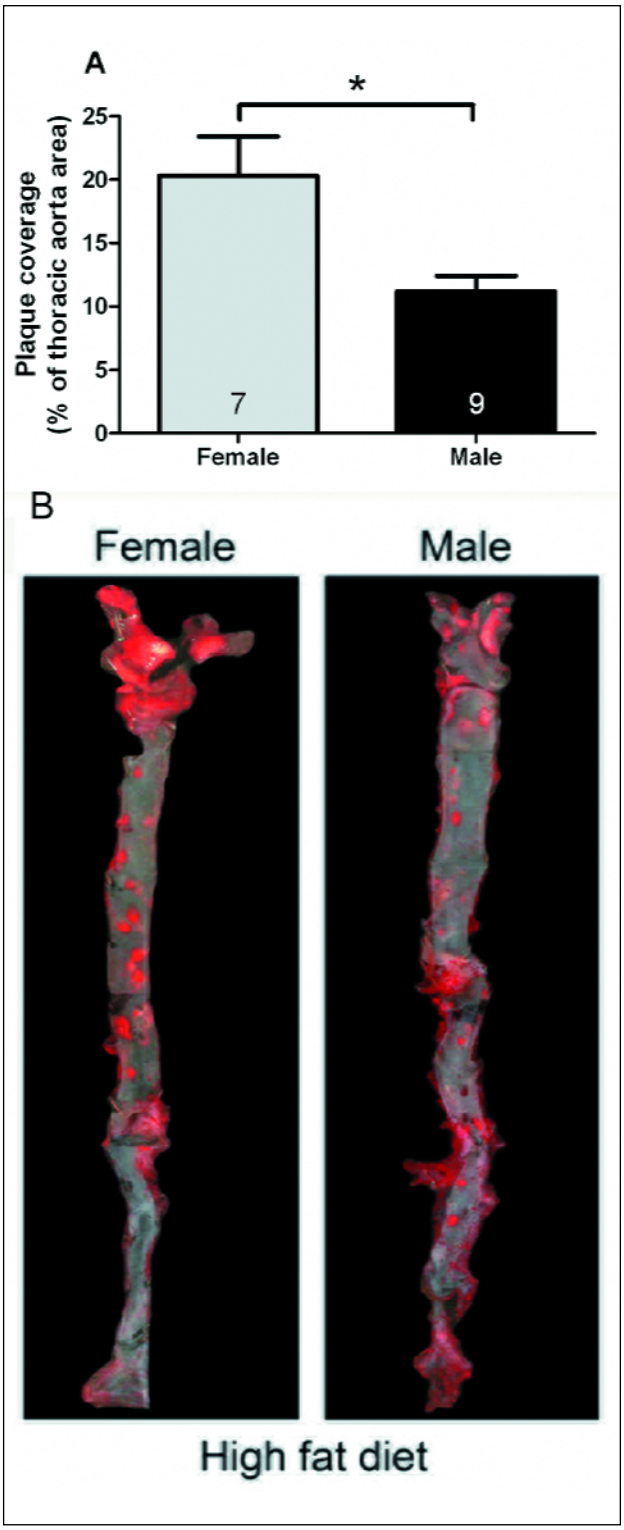

Fig. 1. The effect of gender on the development of aortic atherosclerotic lesions in ApoE$^{-/-}$ mice maintained on a high fat diet for 3 months. At necropsy, the aorta from the root to the iliac bifurcation was dissected free, fixed overnight, spread, and stained with Sudan IV. (A) Quantification of the digital images of en face preparations representing percent of total area of the arch and thoracic aorta covered by plaque. The data presented are mean±SEM. The number of mice used per group is given in each bar. * $p<0.05$, when compared to males. (B) Representative en face preparations of aortas from age-matched female and male ApoE$^{-/-}$ mice on a high fat diet for three months.

whether a disparity in the systemic production of TXA_2 or PGI_2 is associated with the increased progression of atherosclerosis in females, we evaluated the urinary levels of 11-dehydro thromboxane B_2 and 2,3-dinor-6-keto prostaglandin $F_{1\alpha}$, respectively, as indices of the systemic production of TXA_2 and PGI_2. The results presented in *Fig. 3* shows that the production of PGI_2 by female ApoE$^{-/-}$ mice is significant lower than males. In contrast, female ApoE$^{-/-}$ mice produced up to 15 fold more TXA_2 than males. The patterns of increased 11-dehydro thromboxane B_2 in urine from female mice as determined by EIA were further confirmed by gas chromatography/mass spectrometry, which showed at least 5.5 times higher production in females than in males (data not shown). It is noteworthy that markedly elevated levels of 11-dehydro thromboxane B_2 was also present in the urine samples of wild type females maintained on either a normal or the high fat diet (data not shown).

Serum chemistry, lipids, and 17-β estradiol profile reveal gender differences

Serum chemistry, lipids, and 17-β estradiol profile in ApoE$^{-/-}$ male and female mice as well as values for normal mice are presented in *Table 1*. The lipid profile indicates that male ApoE$^{-/-}$ mice have significantly elevated levels of total serum cholesterol with slightly elevated levels of triglycerides, HDL, LDL, and VLDL compared to females. Although, the total cholesterol is found to be lower in females, the cholesterol/HDL ratio tends to be higher due to the slight decrease in HDL levels. Interestingly, the serum lipase level was approximately 30% higher in females. Data presented in *Table 1* also indicate that in comparison to normal mice, glucose, cholesterol, triglycerides, LDL, and total cholesterol/HDL ratio are greatly elevated in both genders of ApoE$^{-/-}$ mice fed the high fat diet. The ALT level is markedly higher in male ApoE$^{-/-}$ mice.

Estrogen is known to be atheroprotective in both female and male ApoE$^{-/-}$ mice (16). In order to determine whether changes in estradiol levels are associated with the magnitude of atherosclerosis lesions, the serum levels of estradiol were analyzed. As shown in *Table 1*, the serum estradiol levels in ApoE$^{-/-}$ females were approximately 41% higher than males. Although elevated levels of serum estradiol were expected in females, the results were not found to be statistically significant. The mean serum testosterone level in four male ApoE$^{-/-}$ mice was found to be 2.3 ng/mL±0.9. Although the levels of testosterone in female ApoE$^{-/-}$ mice were not determined in this study, both ours and the published values for the males presented in *Table 1* are markedly higher than the reported value of 0.15 ng/mL for ApoE$^{-/-}$females (17).

Immunohistochemical staining of aortic tissues for T cells, mast cells, and macrophages reveals no gender-related differences in their recruitment

To determine the relative presence of T cells, mast cells and macrophages in atherosclerotic lesion area, immunohistochemical analyses were performed using antibodies to CD3, CD117 and Mac-3, respectively, (*Table 2*). Interestingly, no apparent differences in T cells, mast cells, and macrophages were noted between genders. However, in comparison to WT mice, an increase in the number of T cells and macrophages were noted in aortic tissues of ApoE$^{-/-}$ mice of both genders. No change in mast cell numbers was noted between WT and ApoE$^{-/-}$ mice.

Table 1. serum chemistry, lipid profile, and 17-β-estradiol levels in ApoE$^{-/-}$ mice fed with a high fat diet for three months.

	ApoE$^{-/-}$ Female	ApoE$^{-/-}$ Male	WT Mice (Normal range)
Glucose (mg/dL)	296.8 ± 20.5 (4)	252.8 ± 31.5 (4)	61.0±9.1(4) [a]
BUN (mg/dL)	23.5 ± 4.6 (4)	23.0 ± 2.0 (4)	37.5±5.6(4) [a]
ALT (U/L)	82.0 ± 11.7 (4)*	226.8 ± 54.0 (4)	77.0±21.7(4) [a]
Lipase (U/L)	61.5 ± 7.6 (4)*	42.3 ± 1.9 (4)	43.8±13.4(4) [a]
Tot. Cholesterol (mg/dL)	961.2 ± 48.0 (11)*	1191.9 ± 83.9 (12)	68.0±9.9(4) [a]
Triglyceride (mg/dL)	169.3 ± 41.1 (11)	228.7 ± 31.2 (12)	71.0[b]
LDL (mg/dL)	972.8 ± 20.2 (6)	1084.3 ± 122.8 (7)	13.3[b]
HDL (mg/dL)	17.6 ± 2.0 (7)	23.1 ± 2.6 (8)	79.7[b]
VLDL (mg/dL)	41.7 ± 12.2 (7)	53.7 ± 7.9 (8)	NA[c]
TC/HDL (Ratio)	63.0 ± 9.9 (7)	52.6 ± 6.1 (8)	0.85[b]
β-Estradiol (pg/mL)	29.5 ± 5.0 (5)	20.9 ± 5.1 (4)	47.3[d] ; 27.8[e]
Testosterone (ng/mL)	Not determined[f]	2.3 ± 0.9 (4)	0.5[e]

Values presented are the mean ± SEM with number of samples given in parenthesis. The number of samples varies because not every animal provided enough serum to be analyzed for every parameter, and obtaining values for the lipid profiles were prioritized.
[a] Values for C57Bl/6J WT male mice on a normal diet.
[b] Values obtained from The Jackson Laboratory Physiological Data Summary for C57Bl/6J mice (gender not specified).
[c] Not available from above Data Summary.
[d] Value for female mice in proestrus (ref 51).
[e] Value for male mice (ref 52).
[f] Published values for female and male ApoE$^{-/-}$ mice on a normal diet are 0.15 ng/mL and 1.4 ng/mL, respectively (ref 17).
* $p \leq 0.05$ when compared to ApoE$^{-/-}$ males (Student's t test).

Table 2. Summary of immuno-histochemistry staining for T cells (CD3), mast cells (Giemsa and CD117) and macrophages (Mac-3) at the root of the aorta in wild type and in ApoE$^{-/-}$ mice fed a high fat diet for three months.

	WT Female	WT Male	ApoE$^{-/-}$ Female	ApoE$^{-/-}$ Male
CD3 (% positive cells)	0.1±0.1(2)[α]	0.0±0.1(2)	5.9±1.3(7)*	6.6±2.2(8)*
Mast Cells (n/HPF)	4.6±0.8(2)	2.5±0.7(2)	3.2±0.6(7)	2.8±0.6(8)
Mac-3 (mean intensity)	56.4±0.4(2)	63.8±2.2(2)	103.1±12.7(7)*	118.0 ±14.9(8)*

[α] Each value presented is the mean ± SEM with number of animals given in parenthesis. From three to forty regions measuring 18,000 to 19,000 μm^2 were selected and counted by ACIS^{tm} on slides from each animal to determine the mean percent positive cells for CD3. Mast cells were quantified by averaging the number of CD117 and Giemsa positive cells in five high power fields. Mac-3+ cells were quantified by measuring the intensity of the stain by ACIS^{tm} in one to 26 regions of similar size. * $p \leq 0.05$ when compared to Wild Type control of the same gender (Student's t- test).

DISCUSSION

Atherosclerotic cardiovascular disease is an inflammatory process which is more prevalent in men than in women of child-bearing age. However, the risk level for cardiovascular disease increases in women after menopause. This evidence led to the hypothesis that estrogen plays an atheroprotective role in women during their child-bearing age. However, due to secondary complications of estrogen supplementation in post-menopausal women and the lack of sufficient supporting data, the cardioprotective potential of estrogen remains a continuing question. The present report demonstrates that the female ApoE$^{-/-}$ mice develop atherosclerotic lesions more aggressively than males which are in contrast to what is seen in humans. Although a variety of gene knockout mouse models are used for evaluating atherosclerosis, it should be recognized that all these models many not present the true characteristics of human disease and the atherosclerotic lesions are developed in different vessel types and locations (18), suggesting different mechanisms.

The ApoE$^{-/-}$ mouse is an extensively used animal model to evaluate the pathophysiology of atherosclerosis (19-21). Although these animals develop aortic atherosclerosis spontaneously when maintained on a normal rodent diet, the disease progression is accelerated by the high-fat regimen (22). Most studies employed adult male ApoE$^{-/-}$ or Ldlr$^{-/-}$ mice to study the progression of atherosclerorosis to avoid potential variability due to cycling estrogen surge in females. The results presented in this report demonstrate that areas of atherosclerosis lesions in the aortas as well as the plaque size are significantly higher in female ApoE$^{-/-}$ mice on a high fat diet. Our results showing increased atherosclerosis progression in female ApoE$^{-/-}$ mice are in agreement with previous reports showing increased atherosclerotic lesions in both ApoE$^{-/-}$ and Ldlr$^{-/-}$ mouse models of C57Bl/6 lineage (23-25). This gender difference in atherosclerotic progression is opposite to what has been described for the human disease. The underlying risk factors of female ApoE$^{-/-}$ mice for increased atherogenic potential are not well-defined. In our study the serum lipid and estradiol profiles did not offer an explanation for the increased progression of atherosclerosis in the ApoE$^{-/-}$ females (*Table 1*).

Thromboxane A_2 and PGI_2 are products of the cyclooxygenase pathway, and have been implicated in the process of atherosclerosis in humans (12, 13) and mouse models (15, 26). In addition, recent reports have revealed that inhibition of 5-lipooxygenase activating protein (FLAP) attenuates the progression of aortic atherosclerosis in ApoE/Ldlr double knockout mouse models (27, 28). It is generally agreed that PGI_2 retards and TXA_2 enhances the initiation and progression of

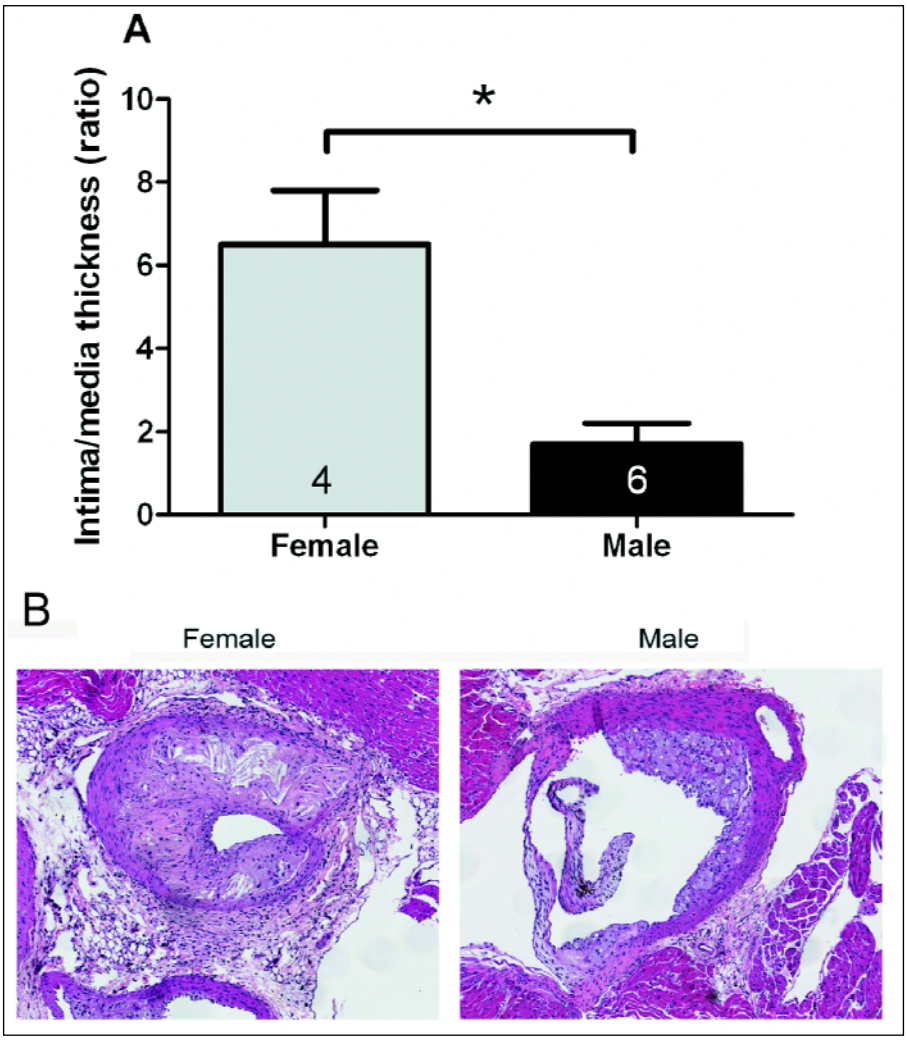

Fig. 2. The effect of gender on the ratio of intima/media thickness of lesions in the aortic root of ApoE$^{-/-}$ mice fed the high fat diet for 3 months. (A) H&E stained cross sections were imaged and the thickness of intima and media was measured at the thickest part of plaque. The data presented are mean±SEM. The number of mice used per group is given in each bar. * $p<0.05$ when compared to males. (B) Representative H&E stained cross sections at the root of the aorta in a female and a male ApoE$^{-/-}$ mouse sacrificed after three months on a high fat diet.

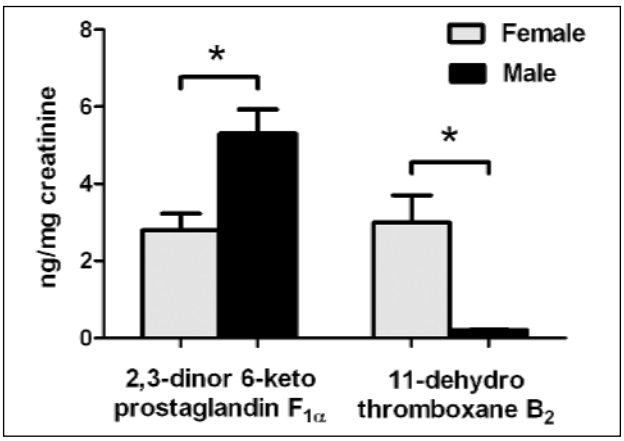

Fig. 3. The effect of gender on the levels of 2, 3-dinor-6-keto prostaglandin $F_{1\alpha}$ and 11-dehydro thromboxane B_2 in 24-hour urine samples of ApoE$^{-/-}$ female and male mice after a 3-month high fat diet regimen. The data presented are as mean±SEM with four animals in each group. * $p<0.05$, when compared to males

atherogenesis through: (1) their vasodilatory or vasoconstrictive properties, respectively, (2) modulation of platelet aggregation, and (3) alteration of leukocyte-endothelial cell interactions (15). Since TXA_2 and PGI_2 exert opposing effects on the endothelium to maintain normal vascular functions, a tightly regulated PGI_2 /TXA_2 homeostasis is critical for the prevention of cardiovascular disease. We observed significant gender disparities in plaque development and urinary levels of 11-dehydro TXB_2 and 2, 3-dinor-6-keto $PGF_{1\alpha}$. ApoE$^{-/-}$ females fed the high fat diet for 3 months, produced up to 15-fold higher TXA_2 (F=2.99 *vs*. M=0.20; ng/mg creatinine) and 50% lower PGI_2 (F=2.8 *vs*. M=5.3 ng/mg creatinine) than males. The increase in urinary 11-dehydro TXB_2 was also evident in WT female mice maintained on normal diet and the high fat diet regimen did not significantly alter TXA_2 production (data not shown). Babaev *et al*., (29) found similar levels of 11-dehydro-TXB_2 in male Ldlr$^{-/-}$mice; and Yan *et al*., (30) reported female ROMK null mice to produce 3 times as much urinary TXB_2 as did males, and almost twice as much in the WT controls of the Black Swiss lineage. Our results suggest that the markedly elevated levels of TXA_2 production together with the reduced PGI_2 production and resultant shift in equilibrium of TXA_2 and PGI_2 is a proatherogenic risk factor in ApoE$^{-/-}$ female mice.

It is well-recognized that total cholesterol levels in the serum are elevated in atherogenic mouse models maintained on a high fat regimen (31, 32). However, the hypercholesterolemia is not necessarily correlated with plaque development (33, 34). In this study, although the total serum cholesterol levels in ApoE$^{-/-}$ females were significantly lower than males, females developed more plaques than males. However, in comparison to males, slightly lower levels of HDL and significantly elevated levels of lipase in the serum were noted in females (*Table 1*). In agreement with our finding, Ishida *et al*. (35) have documented

an inverse relationship between endothelial lipase and serum HDL, and attributed the enhanced atherogenesis to endothelial lipase through monocyte recruitment and cholesterol uptake. Endothelial lipase has also been recognized as an important modulator of HDL levels in the mouse (36-38). Furthermore, lipoprotein lipase has been proposed as one of the key proteins involved in the retention of LDL and VLDL in the arterial intima, by enhancing their adherence to the extracellular matrix (39). Although the source of elevated serum lipase in females is unknown, the relatively higher serum lipase and lower HDL levels may serve as additional risk factor in female ApoE$^{-/-}$ mice.

Both estrogen and testosterone have been found to be protective against the atherosclerotic process in ApoE$^{-/-}$ mice (16, 17, 33, 40). In the present study, although the serum estradiol levels in ApoE$^{-/-}$females were found to be 41% higher than in their male counter parts, the difference was not found to be statistically significant probably due to the relatively smaller sample size. It is noteworthy that such a narrow margin of difference in serum estradiol levels between female and male ApoE$^{-/-}$ mice (33 pg/mL and 24 pg/mL, respectively) has been reported previously (17). It should be emphasized that in spite of the higher serum levels of estradiol in ApoE$^{-/-}$ female mice noted in this study, they developed more severe atherosclerotic lesions than males.

Estradiol generated from testosterone by the enzyme aromatase has been shown to reduce atherosclerosis progression in male Ldlr$^{-/-}$ mice (41). The serum testosterones levels reported in the literature for male ApoE$^{-/-}$ mice (17) as well as for the ApoE$^{-/-}$ males we tested after the high fat regimen are 10 to 15 times higher than that in females (*Table 1*). Since the serum testosterone levels are inherently higher in males, a consistent level of estradiol production *via* aromatase may provide some atheroprotection in males. Thus, it is conceivable that any estrogen-mediated atheroprotection in female ApoE$^{-/-}$ is possibly out-weighed by the overproduction of such proatherogenic agents as TXA_2. However, Villablanca *et al.* (34) reported that estradiol acting *via* estradiol receptor-α induces early atherogenesis in males in the Paigen diet-induced mouse model of atherosclerosis. Since the atheromodulatory effects of estrogen are mediated *via* estradiol receptors in the vessels, it is possible that these receptors are differentially expressed in ApoE$^{-/-}$ males and females. It should be noted that estrogen is known to enhance the production of TXA_2 in female rat aorta by upregulating the expression of COX_2 and TXS in both vascular endothelium and smooth muscle cells (42). In the present study, despite the slight increase in serum estradiol levels, urinary TXA_2 levels were markedly higher in ApoE$^{-/-}$ female mice. Tamoxifen, an estrogen receptor antagonist, has been reported to act as an antiatherogenic and cardioprotective agent in mouse models (5, 6) and in humans (7-10), which raises questions on the role of estrogen in cardiovascular disease. However, in a separate study, neither tamoxifen nor toremifene altered the production of PGI_2 and TXA_2 or their ratio in humans as assed by the urinary excretion of their metabolites (43). In this regard, further studies using overiectomized ApoE$^{-/-}$ females with and without estrogen supplementation, as well as those treated with Tamoxifen are warranted to test the direct association between estrogen and prostanoid homeostasis in the progression of atherosclerosis. These experiments are beyond the scope of this study.

Progression of atherosclerosis involves increased recruitment of macrophages, T and B lymphocytes and mast cells (44). Together, they are thought to promote atherogenesis by releasing proinflammatory cytokines, chemokines, and proteases (45, 46). In support of the concept of the recruitment of inflammatory cells into atherosclerotic lesions, we observed increased number of macrophages and T lymphocytes in the aortic tissues of ApoE$^{-/-}$ mice with atheromas in comparison to WT controls. Mast cells seemed to be present in equivalent numbers in both genders of WT and ApoE$^{-/-}$ mice. In our study, the number of macrophages, T lymphocytes and mast cells were comparable in the aortic tissues of male and female ApoE$^{-/-}$ mice. It is noteworthy that Caligiuri *et al*. (47) reported increased number of CD4+ T cells in the spleen of young female ApoE$^{-/-}$ mice and increased atherosclerotic lesions compared to age matched males.

In summary, the present study demonstrates that female ApoE$^{-/-}$ mice are prone to develop increased atherosclerotic lesions in comparison to males. The increased atherogenic activity in females was found to be associated with markedly increased production of TXA_2 and decreased production of PGI_2. Although the serum cholesterol and triglyceride levels were not correlated with the enhanced progression of plaque development in females, an association was noted with the elevated level of serum lipase and lower HDL levels. It should be emphasized that, despite the availability of a large volume of data on putative risk factors of cardiovascular disease, little is known on the gender differences on the regulation of the expression of novel genes or metabolic products that modulate atherosclerotic progression. In this regard, the role of hyperhomocysteinemia (48) and β-adrenergic receptor-activation associated endothelial dysfunction (49) are of particular interest. Furthermore, the recent study demonstrating differential expression of genes involved in lipid metabolism and tissue architecture in ApoE$^{-/-}$ mice after a high fat regimen (50), suggest the importance of these gene products in atherogenesis. Thus, future studies should evaluate the gender differences on the expression of these candidate genes and other novel risk factors to further delineate the mechanism of increased atherosclerosis in female ApoE$^{-/-}$ mice.

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Conflict of interests: None declared.

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