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Differential expression of genes in the calcium signaling pathway underlies lesion development in the *LDb* mouse model of atherosclerosis

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Abstract

Objective—Atherosclerosis is influenced by the interaction of environmental and genetic susceptibility risk factors. We used global microarray expression profiling to investigate differentially regulated genes in aorta during development of atherosclerosis in a susceptible genetically modified mouse model in response to the interaction between risk factors including hyperlipidemic genotype, shear stress, diet, and age.

Methods and Results—In this study we investigated transcriptional changes in lesion-prone and lesion-resistant regions of aortas in genetically modified mice lacking both genes of the LDL receptor and the apolipoprotein B mRNA editing enzyme (*LDb*; *Ldlr*^{−/−}*Apobec1*^{−/−}). Risk factors including hyperlipidemic genotype (*LDb* vs. *C57BL/6* wildtype), shear stress (lesion-prone vs. lesion resistant aortic regions), diet (chow vs. Western high-fat), and age (2-months vs. 8-months) were studied. We hybridized aortic RNA samples with microarray chips containing probes for 45,000 mouse genes and expressed sequence tags (ESTs). Overall, the differentially expressed genes were components of 20 metabolic and physiological pathways. Notably, calcium signaling is the major pathway identified with differential regulation of 30 genes within this pathway. We also found differential expression of calcium signaling genes in cultured primary endothelial cells from lesion-prone and lesion-resistant arterial regions (*LDb* mice vs *C57BL/6* controls), providing further support for involvement of calcium signaling in the pathogenesis of atherosclerosis. Moreover, we demonstrated protein expression of genes in the calcium signaling pathway using Western blot analysis and immunofluorescence.

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Conflict of Interest

The authors have no conflicts of interest to disclose

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Conclusions—Our results suggest that calcium signaling may play an important role in regulation of genes expressed in aorta during development of atherosclerosis. Calcium signaling may act via mechanistic responses to genetic, mechanical, and environmental insults that trigger an imbalance of intracellular calcium homeostasis, resulting in altered biological processes leading to lesion development.

Keywords

atherosclerosis; cDNA microarray; aorta; risk factors; calcium signaling pathway

1. INTRODUCTION

Atherosclerosis is the primary cause of coronary artery disease (CAD) and the leading cause of death in North America. As the genetic details of CAD pathways are being elucidated, it is clear that the development of atherosclerosis involves cascades of molecular interactions between multiple genes and environmental factors, ultimately resulting in the development of pathological lesions. Studies have identified numerous risk factors that contribute to CAD such as hyperlipidemia, aging, diet, smoking, type 2 diabetes, and hypertension. In addition, it is now recognized that shear stress related to blood flow is an independent risk factor in the development of atherosclerosis^{1, 2}. This concept is based on the observation that atherosclerotic plaques occur preferentially in areas such as the inner curvatures of coronary arteries or near bifurcations, where shear stress is oscillatory^{3–5}. Although previous studies have investigated changes in gene expression in CAD with regards to shear stress⁶, age⁷, genetic strain⁷, and diet⁸, we know of no gene expression study that examined the combined effects of these CAD risk factors on atherogenesis.

We designed this experimental study to investigate the effects of these risk factors on differential gene expression in aorta using a genetic modified mouse model (LD_b) that has demonstrated susceptibility to atherosclerosis. Teng and coworkers^{9, 10}, as well as others¹¹, developed the LD_b mouse model (*Ldlr*^{−/−}*Apobec1*^{−/−}) by deleting the genes encoding the LDL receptor (*Ldlr*) and the apolipoprotein B mRNA editing enzyme (*Apobec1*). In contrast to wild type mice, the phenotype of the LD_b mice closely mimics humans with hyperlipidemia characterized by increased plasma levels of LDL cholesterol (LDL-C) and decreased levels of HDL-C. In addition, these LD_b mice spontaneously develop severe atherosclerotic lesions, even when fed on a normal chow diet. Teng and coworkers⁹ performed global profiling of hepatic gene expression in LD_b mice using microarrays that demonstrated differential expression of genes in pathways for inflammation and calcification. These results were further supported by detection of marked amounts of macrophages and calcification in the aorta of LD_b mice⁹. In the present study, we analyzed global gene expression of aorta in LD_b mice under the influence of four risk factors including hyperlipidemic genotype, diet, aging, and shear stress. We showed that the calcium-signaling pathway is the major controlling factor underlying atherosclerotic lesion development.

2. MATERIALS AND METHODS

2.1 Study Design

The atherosclerosis susceptible LD_b mouse model (*Ldlr*^{−/−}*Apobec1*^{−/−} on a C57BL/6 genetic background) was generated in Dr. Teng's laboratory as previously described^{9, 10}. Figure 1A shows the experimental design to evaluate aortic differential gene expression influenced by genetic modification (LD_b vs. C57BL/6 wild-type), diet (chow vs. Western-high fat diet), age (2-months vs. 8-months), and shear stress (lesion-prone vs. lesion-resistant). Male LD_b mice and C57BL/6 control mice (n = 16 per group) were weaned at 4 weeks of age. They were divided and fed with either a chow diet (n=8) or a Western-high fat diet (n=8) (TD 88137,

Harlan Teklad, Madison, WI) for a designated time of 1-month or 7-months. From each diet group, four mice were sacrificed at one-month after feeding (2-months-of age) and the other four mice were sacrificed at seven months after feeding (8-months of age). The aorta from each animal was divided into lesion-prone segments of aortic arch (LP) and lesion-resistant segments of the thoracic region (LR) as shown in Fig. 1B for microarray gene expression analysis (Fig. 1A). All animal experiments were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee from the University of Texas Health Science Center at Houston.

2.2 RNA preparation, microarray hybridization, and array analysis

The aorta of each mouse was perfused with cold PBS through the left ventricle of the heart. The aorta was cleaned of adventitial fat and connective tissues. The lesion-prone region (LP) was dissected from the ascending aortic root to the fourth rib where the region extended into the thoracic portion. The lesion-resistant region (LR) was the adjacent segment located from LP to the branching of the renal artery. Each segment of the aorta was flash frozen in liquid nitrogen. We extracted total RNA from each tissue segment using Trizol reagent (Invitrogen), followed by Qiagen RNeasy Mini Kit (Qiagen). RNA concentrations were determined by spectrophotometry, and RNA integrity was evaluated on a 1% agarose gel. The yield of total RNA was 3–10 µg from the LP segment and 1–5 µg from the LR segment.

We used 100 ng of total RNA from LP or LR segments to synthesize biotinylated labeled cRNA probe as described in Two-Cycle Eukaryotic Target Labeling for GeneChip Expression Analysis (Affymetrix, Santa Clara, CA). We hybridized 5 µg of fragmented biotinylated cRNA to an Affymetrix Test 3 control chip to assess the probe quality. We then used 15 µg of fragmented cRNA probe to hybridize to an Affymetrix Murine 430, 2.0 microarray chip (Affymetrix Murine 430, 2.0 chip interrogates approximately 45,000 mouse genes and ESTs) at 45°C for 16 hours, followed by wash cycles and stain procedure as described in the EukGE-WS2v5 protocol. The fluorescent hybridization signals were scanned and captured on an Affymetrix Scanner 3000 at Baylor College of Medicine Microarray Core Facility and the fluorescent signal intensities on the array were analyzed using the Gene Chip Operating System (GCOS; Affymetrix) to generate image (.dat) files.

2.3 Microarray analysis

All 64 .dat image files were imported into the dChip software (www.dChip.org)¹² for normalization and analysis. The background artifacts were manually inspected and removed. All the 64 chips were then corrected for local background correction (dChip, ID #6444). After the adjustment, the data was normalized using two methods implemented in the dChip analysis software; a LOWESS algorithm as implemented in the invariant set method (dChip)¹³ and a quantile normalization (RMA) method¹⁴. Image intensities above a 20,000 cut-off value were excluded, single and array outliers were regarded as missing values or blank. Probe intensities were extracted and summarized in a Model Based Expression Index (MBEI) file. The average of the median intensities of the 64 chips was 125±45. The average percentage of probes that were detected or present was 48%±11. The average array outlier was 4.8%.

We used t-test for analyzing differential gene expressions. T-test is a robust parametric analysis and is a preferred method when comparing the difference between the mean of two groups with independent variables with normal distribution. The t-statistic in the dChip software was used to compute the difference between groups and its p-value was computed based on the t-distribution, and the degree of freedom was set according to Welch modified two-sample t-test (dChip.org). For all t-test comparisons outlier expression values were treated as missing data in the comparison analysis, we assumed two-tail t-test with equal variance, except for shear

stress comparison between lesion-prone and lesion-resistant a paired t-test with equal variance was used instead.

Fold change in gene expression was defined as the average normalized expression signals of mice from the “treatment” group divided by the average normalized expression signal of mice from the “untreated” group (e.g. age=8-months/2-months, diet=high-fat/chow, genotype=LDb/C57, and shear stress=LP/LR). To adjust for multiple comparisons, we randomly permuted the results (100 times each) to estimate the mean empirical false discovery rate (FDR)^{15, 16}, and chose a FDR of = 0.15 to determine significance of differentially expressed genes. Candidate genes meeting these criteria were imported for pathway analysis.

2.4 Pathway analysis

We submitted our lists of differentially expressed genes and their fold changes from the t-tests for analysis by Pathway Express (<http://vortex.cs.wayne.edu>). This pathway analysis is free to the public, offers easy and fast data handling, and the pathway networks have been curated. Pathway Express Software used the Kyoto Encyclopedia of Genes and Genomes (KEGG) database^{17, 18} to search for functional pathways that are significantly over-represented by differentially expressed genes. The algorithm of the Pathway Express used fold-change of expression for each input gene, positions of genes in pathway hierarchies, and the proportion of genes in the pathway that are differentially regulated to calculate a perturbation factor (PF), which generated the significance of pathways^{19, 20}. Moreover, PF values were used to derive significance levels to rank the significant over-represented pathways in each risk factor.

2.5 Validation of microarray results by quantitative real-time PCR of calcium signaling gene expression

A set of 17 genes was chosen from 30 differentially expressed genes in the calcium signaling pathway for quantitative real-time PCR (RT-PCR) to validate the differential expression determined by microarray^{21, 22}. Oligonucleotide primer sets for each gene were designed using Primer Express software (Applied Biosystems). RT-PCR was performed using the SYBR Green master mix reagent (Applied Biosystems) in an ABI Prism 7900HT Sequence Detection System. Briefly, cDNA was produced from each sample using the Achieved cDNA kit (Applied Biosystems). The optimized concentration of each gene-specific primer set was determined as shown in (Supplement Table 1). We used a standard curve method to quantify gene expression, and the relative expression of each gene was normalized with 16S RNA. The PCR reaction was performed using the default protocol: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 60 sec at 60°C. The dissociation curve was obtained using a thermal regime of 15 sec at 95°C, 15 sec at 60°C, and 15 sec at 95°C.

2.6 Isolation and culture of primary vascular endothelial cells

Primary endothelial cells were cultured from lesion-prone and lesion-resistant arterial sections of C57BL/6 and LDb mice at 8-months of age. Briefly, aorta was cleaned as described above. Aortic segments (3 mm in length) were placed onto a 35-mm matrigel culture plate containing endothelial media (DMEM media supplemented with 15% FBS, heparin 90 µg/ml, ECGS 60 µg/ml, and 1% Antibiotic-Antimycotic)²³. After approximately 4–7 days of growth, primary endothelial cells were removed from matrigel plates using Dispase, plated onto 0.2% gelatin-coated culture dishes, and expanded in endothelial media.

2.7 Characterization of primary vascular endothelial cells

Uptake of Dil (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate)-labeled Acetyl-LDL—We plated mouse primary vascular endothelial cells obtained from C57BL/6 and LDb mice as described above and control human EA.hy926 cells

(kindly provided by Dr. CoraJean S. Edgell, University of North Carolina, Chapel Hill, NC) in a 0.2% gelatin coated chamber slide overnight. Cells were incubated in media (300 μ l) containing 10 μ g/ml DiI-Acetyl-LDL (Invitrogen) at 37°C for 6 h. The media was removed; the cells were washed three times with PBS, followed by fixing with 4% buffered formalin phosphate (Fisher Scientific) for 10 min. The slides were mounted with mounting medium containing DAPI (Vector). The image was examined using a Zeiss Axioskop fluorescence microscope (Zeiss Observer.D1m). A Texas Red filter set was used to image DiI.

Immunofluorescence analysis—We cultured human aortic smooth muscle cells (Cambrex), EA.hy926, and primary vascular endothelial cells from C57BL/6 and LDb in a 0.2% gelatin coated chamber slide overnight. The cells were washed with PBS three times, and fixed with 4% buffered formalin phosphate for 10 min. We then incubated the cells with PBS containing 0.25% Triton X-100 to permeabilize the cells, followed by washing with PBS three times. The slides were incubated with 1% BSA in PBST (0.1% Tween-20) for 30 min to block non-specific binding. The cells were then incubated with rabbit anti-CD31 at 1:100 (Fisher Scientific) or rabbit anti- α actin at 1:100 (Abcam) at 4°C overnight. Next day, the cells were washed and incubated with Alexa-594 conjugated secondary goat anti-rabbit IgG at 1:300 for 1h at room temperature. After washing off the secondary antibody, all the slides were examined using a Zeiss Observer.D1m fluorescence microscope with Texas Red and DAPI filters. Control slides without primary or secondary antibodies were performed at the same time.

2.8 RNA extraction and RT-PCR quantification of calcium signaling gene expression in primary vascular endothelial cells

RNA was extracted from lesion-prone and lesion-resistant primary endothelial cells using Trizol (Invitrogen). After treatment with DNase I (Ambion), endothelial cell RNA samples were used for cDNA synthesis with a high-capacity cDNA reverse transcription kit (Applied Biosystems). Quantitative RT-PCR assays of gene expression for the 17 genes selected from the calcium signaling pathway were performed using the SYBR Green master mix reagent (Applied Biosystems) with the ABI Prism 7900HT Sequence Detection System as described above.

2.9 Western blot analysis

The whole aortas from C57BL/6 and LDb mice at 2- and 8-months of age fed a chow diet were collected as described in Section 2.2 and snap frozen until analysis. The frozen aorta was homogenized in 0.25 ml ice-cold M-PER Mammalian Protein Extraction Reagent (Pierce) containing protease inhibitors (Roche Molecular Biochemicals). After homogenization, tissue homogenate was kept on ice for 30 min, followed by centrifugation at $10,000 \times g$ for 10 min at 4°C. The supernatant was kept at -80°C until analysis.

Proteins from cultured primary endothelial cells of Lesion-prone (LP) and Lesion-resistant (LR) regions were extracted using the same buffer. Protein concentrations were determined using the Bradford assay (Bio-Rad).

We selected certain genes in the calcium signaling pathway for Western Blot analysis. We used 15 μ g protein content to separate proteins by either 6% or 15% SDS-PAGE according to their molecular weight. The proteins were transferred to Immobilon P membranes (Millipore). The membranes were incubated with the following antibodies (Abcam) to detect the presence of each protein: rabbit monoclonal to Calmodulin (ab45689) at 1:1000, mouse monoclonal to SERCA2 ATPase (ab2861) at 1:1000, rabbit polyclonal to GNAQ (ab75825) at 1 μ g/ml, rabbit monoclonal to myosin light chain kinase (ab76092) at 1:4000, rabbit polyclonal to prostaglandin E receptor EP3 (ab21227) at 1:1000, and mouse monoclonal to Gapdh

(Chemicon International) at 1:3000. The membranes were subsequently incubated with secondary antibody (Invitrogen) at 1:3000 dilution of either Alexa680 conjugated goat-anti-rabbit IgG or Alexa680 conjugated goat -anti-mouse IgG. Each protein was detected and quantified using an Odyssey infrared imaging system (LI-COR). Each protein was normalized with the corresponding Gapdh value. The normalized protein value was used to perform statistical analysis to compare the difference between the groups of aorta-LDb vs. aorta-C57BL/6, or LDb-LP vs. C57BL/6-LP, or LDb-LR vs. C57BL/6-LR. We used unpaired t-test with Welch's correction implanted in the GraphPad Prism, ver 5.0 (GraphPad Software, Inc., La Jolla, CA), the p value < 0.05 was significant.

2.10 Immunofluorescence analysis on Aortic Sinus

Cryosections (5 micron) of aortic sinus from 8-months of age C57BL/6 and LDb mice fed a chow diet were fixed in 4% buffered formalin phosphate for 10 min at room temperature. After washing, the sections were incubated with 1% BSA PBS-Tween 20 for 1h at room temperature to block nonspecific binding. The sections were then incubated at 4°C overnight with the following primary antibodies, 1:100 dilution of goat anti-CD11b (Pharmingen), 1:100 dilution of goat anti-CD68 (Santa Cruz Biotech), 1:50 dilution of mouse anti-SERCA2 (Abcam), 1:100 dilution of rabbit anti-Calmodulin (Abcam), 1:100 dilution rabbit anti-GNAQ (Abcam), 1:100 dilution rabbit anti-myosin light chain kinase (Abcam), and 1:100 dilution rabbit anti-prostaglandin E receptor EP3 (Abcam). After washing, the slides were incubated with 1:150 Alexa-594 or Alexa-488 conjugated corresponding secondary antibodies (Invitrogen) for 1h at room temperature. The slides were subsequently washed and mounted with mounting medium containing DAPI (Vector) and examined using a Zeiss Axio observer.D1m fluorescence microscopy with DAPI, FITC and Texas Red filters. Control slides without primary or secondary antibodies were performed at the same time.

3. RESULTS

3.1 Metabolic and biochemical characteristics of LDb mice fed chow and high-fat diets

Table 1A shows the plasma parameters for the wild-type C57BL/6 and LDb male mice on regular chow diet or Western high-fat diet. All the mice were weaned at one-month of age and entered the study on a chow or a high-fat diet for the designated time. The body weight of each strain increased steadily from 2 months to 8 months of age, and there were no differences between the two genotypes. The mice on high-fat diet became heavier than the mice on chow diet for both C57BL/6 and LDb mice only at 8-months of age. The levels of plasma cholesterol and triacylglycerol in LDb mice were at least 5 times higher than C57BL/6 mice. As shown by FPLC distribution profiles, similar to hyperlipidemia patients LDb mice had elevated VLDL and LDL cholesterol, whereas most of the plasma cholesterol in C57BL/6 mice was in the HDL fraction (Fig. 1C and Table 1A). In contrast, LDb mice had approximately 20% of plasma cholesterol in the HDL subfraction. Thus, LDb mice have a similar lipoprotein profile as hyperlipidemia patients.

There were no obvious effects on plasmid lipid levels after feeding a high-fat diet for one month to either C57BL/6 or LDb mice (Table 1A). However, after 4 months on a high-fat diet, plasma cholesterol levels increased ~3-fold compared to the chow diet in both strains. The plasma triacylglycerol levels did not change much in C57BL/6 mice, but increased markedly (> 3-fold) in LDb mice after feeding a high-fat diet for over 4 months. The high-fat diet decreased the ratio of HDL cholesterol to total cholesterol (HDL-C/TC) in C57BL/6 mice (decreased 20% after 4 months and 50% after 7-months), suggesting that long-term feeding of the high-fat diet increased levels of VLDL and LDL cholesterol and decreased HDL levels in the C57BL/6 mice.

3.2 Atherogenesis in LDb mice

We used the en-face technique to quantify atherosclerotic lesions in the whole aorta to assess lesion development in the mice. The percentage of lesions for the whole aorta in both strains were quantified as shown in Table 1B. As expected, the control C57BL/6 mice on both diets did not develop any lesions throughout the course of the experiment. In LDb mice, there were no detectable lesions at 2-months of age. However, by 8 months of age, > 20% of the aortic surface was covered with lesions on the chow diet. High-fat diet accelerated lesion development in LDb mice, with > 30% of the aortic surface involved in atherosclerotic lesions. As shown in Figure 1B, the LDb mouse had atherosclerotic lesions clustered in the aortic arch and the vessel branching point to the renal arteries, anatomic regions associated with high shear stress. In this study, we used a microarray expression profiling approach to identify genes and pathways that contribute to development of atherosclerotic lesions via differential gene expression in the ascending aortic arch (lesion-prone region) compared to the descending thoracic aorta (lesion-resistant region).

3.3 Global gene expression analysis

Gene expression in the aorta was compared among four factors including genotype, age, diet, and shear stress. The differentially expressed genes were analyzed using Pathway express software to identify underlying biological pathways. Table 2A shows the top six canonical biological pathways as determined by rank scores. The calcium signaling pathway was ranked the highest with a sum rank score of 0.818, followed by complement and coagulation cascade (0.682), focal adhesion (0.676), phosphatidylinositol signaling system (0.643), cytokine-cytokine receptor interaction (0.530), and MAPK signaling pathway (0.410).

Next, we analyzed the biological pathways of differentially expressed genes for each individual factor. As shown in Table 2A, there were 30 genes that were differentially regulated in the calcium signaling pathway, with highly significant p values for the pathway for genotype ($p=0.0021$), diet ($p=0.0002$), and shear stress ($p=0.00064$). Age did not play a significant role in the pathway of calcium signaling. There were 17 differentially expressed genes in the complement and coagulation cascade, with significant values for all four risk factors. The focal adhesion pathway had 26 differentially expressed genes, with significant p-values for genotype, age, and diet, but not shear stress. The phosphatidylinositol signaling pathway had 12 differentially expressed genes, and was not significant for the diet factor. The cytokine-cytokine receptor interaction pathway had 44 genes represented, with significant p values for age and diet. The MAPK signaling pathway had 19 differentially expressed genes with significant p-values for genotype, diet, and shear stress.

In Table 2B, we list the fold changes for differential expression of 30 unique genes in the calcium signaling pathway for the corresponding risk factors. In the calcium-signaling pathway, genotype was the risk factor that affected the most number of genes, followed by shear stress, age, and diet.

3.4 Quantitative RT-PCR confirmation of microarray results for differentially expressed genes in the calcium signaling pathway

The calcium-signaling pathway was ranked as the most significant pathway in this study with 30 genes that were differentially expressed. We used quantitative RT-PCR to confirm the results of microarray assays for 17 of the differentially expressed genes. The fold changes calculated from RT-PCR assays correlated well with microarray assays (correlation coefficient was 0.82).

3.5 Perturbation of the calcium signaling pathway in endothelium during atherogenesis

To determine whether the calcium signaling pathway plays an important role in vascular endothelial cells, we isolated and cultured primary vascular endothelial cells from lesion-prone and lesion-resistant sections of *LDb* and *C57BL/6* mice. In here, we focused on the 2 factors, genotype and shear stress. As shown in Figure 2, primary endothelial cells from both lesion-prone and lesion-resistant sections of *C57BL/6* mice formed tubular structures. Unlike *C57BL/6* mice, cells from lesion-prone sections of *LDb* mice (mouse # 1703 and 254) were disorganized, whereas cells from lesion-resistant sections formed tubular structures. Thus, the endothelial cells were morphologically altered by the development of atherosclerosis.

To characterize these endothelial cells, we incubated the primary cultured endothelial cells obtained from *LDb* mice and *C57BL/6* mice, as well as the control EA.hy926 endothelial cells with DiI-AcLDL (Supplement Fig. 1A). All of these cells took up acetylated LDL. We hybridized the cells with anti-CD31 (Supplement Fig. 1B), the EA.hy926 endothelial cells and endothelial cells from *LDb* and *C57BL/6* mice stained positive with CD31, whereas aortic smooth muscle cells (AoSMC) stained negative with CD31. We also stained these cells with anti- α -actin (Supplement Fig. 1B), as expected, only AoSMCs were detected positive immunofluorescence signal with α -actin, not endothelial cells. Thus, we confirmed that these primary cultured cells are endothelial cells.

Next, we used RT-PCR to quantify transcripts from the 17 selected genes in the calcium signaling pathway (Table 3) in these endothelial cells. The LP/LR gene expression ratios were altered in primary vascular endothelial cells in both *C57BL/6* mice and *LDb* mice, and during atherogenesis. For example, gene expression of *Ptger3* decreased 3.64-fold in primary endothelial cells (Table 3), corresponding to a decrease from the microarray results ($\downarrow$ 2.45-fold) (Table 2B). *Slc8a1* gene expression decreased 1.65-fold, corresponding to a decrease in the microarray results ($\downarrow$ 2.85-fold) (Table 2B). *Vdac1* gene expression increased 2.86-fold, corresponding to an increase in the microarray results ($\uparrow$ 2.75-fold) (Table 2B). We found good correspondence of differential gene expression in the primary endothelial cells with microarray results for *Mlck* ($\uparrow$ 3.71-fold), *Plcb4* ($\downarrow$ 1.47-fold), and *Pln* ($\uparrow$ 2.86-fold). However, primary endothelial cell expression did not correlate well with microarray results for *Ptgfr* ($\downarrow$ 1.98-fold vs. $\uparrow$ 1.25-fold), *Gnaq* ($\uparrow$ 1.79-fold vs. $\downarrow$ 1.52-fold), and *Atp2a2* (no change vs. $\uparrow$ 2.46-fold). Taken together, these findings of differential gene expression in arterial segments (microarray) and primary vascular endothelial cells (RT-PCR) provide strong evidence for marked perturbation of calcium-signaling in endothelium during atherogenesis.

3.6. Western blot analysis of protein expression

To determine whether the observed differences in gene expression were accompanied by changes in protein abundance, we performed Western blot analysis to estimate the levels of protein expression of five genes (*Gnaq*, *Calm1*, *Atp2a2*, *Ptger3*, and *Mlck*) in the calcium signaling pathway. We compared the protein expression of the whole aorta between control *C57BL/6* and *LDb* mice fed on a chow diet at 2- and 8-months of age (Fig. 3A). There was no significant difference in protein levels of *Gnaq* between control *C57BL/6* and *LDb* mice at 2- or 8-months old. The protein levels of *Calm1* (calmodulin) in *LDb* mice increased significantly at both 2-months and 8-months of age, when compared to *C57BL/6* mice ($p=0.0234$ and 0.0376 , respectively). There was no significant difference in the protein levels of *Atp2a2* (SERCA) at 2-months of age between *LDb* and *C57BL/6* mice. However, at 8-months of age the protein levels of *Atp2a2* (SERCA) in *LDb* mice was significantly increased ($p=0.0287$) in comparison to that in *C57BL/6*. The protein levels of *Ptger3* (EP3) decreased substantially in *LDb* mice at 8-months of age ($\downarrow$ 3.3-fold, $p=0.0131$). In general, the protein expression levels corroborated relatively well with the fold changes in gene expressions as shown in Table 2B; *Gnaq* decreased 1.52-fold in genotype, *Atp2a2* increased 2.46-fold in stress factor, and *Ptger3* decreased 2.45-

fold in stress factor. The exception is the *Mlck* gene. The aortic protein expressions of *Mlck* in *LDb* mice at both 2-months and 8-months of age decreased markedly (~10-fold, $p=0.0015$, 0.0041 , respectively) in comparison with that in *C57BL/6* mice. This finding was opposite to the fold-changes of gene expression of *Mlck*; the gene expression levels increased 8.34-fold in genotype and 3.86-fold in stress factor (Table 2B).

The protein expression levels of primary LP and LR endothelial cells from *C57BL/6* and *LDb* mice were shown in Fig. 3B. In here, we compared the protein expression levels of *LDb* to *C57BL/6* (*LDb/C57BL/6*). The protein expression levels of LP and LR from *LDb* endothelial cells were increased in *Gnaq*, *Calm1*, *Atp2a2*, and *Mlck* genes in comparison with that of *C57BL/6*. These results correlated relatively well with gene expression levels presented at Table 3 column of “during atherosclerosis” ($\uparrow 1.79$, $\uparrow 2.0$, no change, and $\uparrow 3.71$, respectively). However, only the difference of *Calm1*-LR and *Atp2a2*-LR protein between *LDb* and *C57BL/6* derived cells reached significant levels ($p=0.0183$ and $p=0.0495$, respectively). The *Ptger3* protein expressions decreased markedly in *LDb*, compared to *C57BL/6*, which was in the same direction as gene expression levels ($\downarrow 3.64$, Table 3). Taken together, the differential changes of endothelial cells protein expressions of *Gnaq*, *Calm1*, *Atp2a2*, *Ptger3*, and *Mlck* between *C57BL* and *LDb* were similar to that in gene expressions of endothelial cells. Furthermore, except for the *Mlck* protein, the differential changes in endothelial cells proteins were in the same direction as proteins detected in the whole aorta.

3.7. Detection of protein expression in aorta

The differences in *Mlck* protein expression between whole aorta and endothelial cells led us to examine the localization of *Mlck* protein in aorta using immunofluorescence techniques. We performed this study on aortic sinus of *C57BL/6* and *LDb* mice fed a chow diet at 8-months of age. As shown in Fig. 4A, the aortic sinus of *LDb* mouse contained extensive atherosclerotic lesions, whereas the *C57BL/6* mouse was normal. The lesions contained an abundance of monocytes/macrophages that stained strongly with CD11b and CD68 antibodies. Fig. 4B illustrates the protein expression of *Gnaq*, *Calm1*, *Atp2a2* (SERCA), *Ptger3* (EP3), and *Mlck* in aortic sinus. As shown, *Gnaq*, *Calm1*, and *Ptger3* proteins were detected mainly on the luminal layer of the aorta that contains endothelial cells and smooth muscle cells, but were not present within the atherosclerotic lesions. In contrast, *Atp2a2* (SERCA) was detected mainly within the atherosclerotic lesions. Furthermore, *Mlck* expressed vividly in the aorta of *C57BL/6* mouse, but was barely detectable in the *LDb* mouse. This result corresponded well with the protein expression of aorta by Western blot analysis. Taken together, the immunofluorescence studies show that *Gnaq*, *Calm1*, and *Ptger3* proteins are expressed mainly in the outer luminal layer of the aorta, and *Atp2a2* (SERCA) protein was expressed mainly within the atherosclerotic lesions.

4. DISCUSSION

We have previously studied hepatic global gene expression of *LDb* mice (*Ldlr*^{-/-}*Apobec1*^{-/-})⁹. That study revealed differential-expression of calcium signaling genes in the liver influenced by high fat diet. In this study, we investigated genes and pathways in the aorta that contribute to atherosclerotic lesion development in *LDb* mice. We compared gene expression in aortic wall of four risk factors including genotype (*LDb* vs. *C57BL/6* wild-type), age (2-months vs. 8-months), diet (chow vs. Western high-fat), and shear stress (lesion-prone versus lesion-resistant regions). We discovered six biological pathways that were over-represented for differentially expressed genes including calcium signaling, complement and coagulation cascades, focal adhesion, phosphatidylinositol signaling, cytokine-cytokine receptor interaction and MAPK signaling (Table 2A). Among these pathways, the calcium signaling pathway was the most over-represented, with differential gene expression for 30 out of 185

curated calcium-signaling genes in the KEGG pathway (<http://www.genome.jp/kegg/pathway.html>) (Supplement Figure 2 and Table 2B).

Calcium signaling plays a vital role in a wide ranges of metabolic and physiological processes^{24–26}. Concentrations of intracellular calcium must be tightly regulated to maintain a proper balance for cellular function. Elevation or prolonged increases in intracellular concentrations of calcium can lead to irreversible cell death due to impaired mitochondrial function and release of cytochrome C that activates phospholipases, proteases, and endonucleases²⁶. In addition, high intracellular concentrations of calcium are associated with diseases that involve abnormal regulation of cellular processes including inflammation, cell growth and proliferation, thrombosis, oxidative stress, and calcification. Likewise, low intracellular concentrations of calcium are also destructive, leading to activation of stress signaling pathways that activate other genes involved with apoptosis. Many of these cellular processes are involved in development of atherosclerosis, and have been associated with alterations in calcium signaling in the vascular wall. Recent studies have implicated alterations in calcium signaling in macrophage apoptosis induced by oxidized LDL, a process important in formation of necrotic cores that may contribute to vulnerability to plaque rupture^{27, 28}. A recent study by Yuan and coworkers²⁹ that compared inbred mouse strains C57BL/6 and C3H/HeJ revealed that the calcium signaling pathway controls atherosclerosis susceptibility.

Extracellular stimuli initiate entry of calcium ion via activated channels and receptors located on the plasma membrane, and by release of calcium from the intracellular storage compartments (ER/SR and mitochondria) to the cytoplasm to modulate cellular processes. During atherogenesis, gene expression of plasma membrane receptors (Egfr, Ptger3, Ptgrfr, and Ryr2) were all down regulated in primary endothelial cells, as well as the calcium channel *Cacna1c* (Table 3). The protein levels of Ptger3 (EP3) were decreased in LDb aorta (Fig. 3A) and endothelial cells (Fig. 3B). Moreover, gene expression levels (Table 2B and 3) of both *Ip3r1* (regulates the release of Ca⁺⁺ from ER to cytoplasm) and *PLcb4* (regulates *Ip3r1* activity) were also decreased. These results suggest that both Ca⁺⁺ entry and Ca⁺⁺ release that introduce Ca⁺⁺ into the cytoplasm were down regulated.

To achieve low cellular calcium concentration, cells need to sequester cytosolic calcium ions in the mitochondria and ER/SR storage compartments. To sequester Ca⁺⁺ in the ER and mitochondria, the *Pln* gene was up regulated to control the activity of *Atp2a2* (Tables 2B and 3). The aortic *Atp2a2* (SERCA2) protein increased markedly in LDb mice (Fig. 3A and 3B) and it was detected in atherosclerotic lesions (Fig. 4B), which suggests that during atherogenesis *Atp2a2* is actively pumping the Ca⁺⁺ from cytoplasm back to ER to modulate cell injury. SERCA2 has been shown to alter Ca⁺⁺ release in *Apoe*^{−/−} mice before development of atherosclerotic lesions³⁰. The levels of SERCA2 increased in dyslipidemic pigs³¹ and in cells treated with oxidized LDL³². Thus, SERCA2 plays an important role in maintaining Ca⁺⁺ homeostasis, and atherosclerotic lesion development. Other genes were also involved in maintaining Ca⁺⁺ levels; cells can directly pump calcium ions into the mitochondria via the voltage-dependent anion channel 1 (*Vdac1*), which is up-regulated during atherogenesis (Table 3). Cells can inhibit release of calcium ions from mitochondria to the cytoplasm via a family of solute carrier proteins such as *Slc8a1*, which is down-regulated during atherogenesis (Table 3). In addition, *Slc25a4* gene expression was up-regulated in LDb mice, probably to bring Ca⁺⁺ from the cytoplasm to the mitochondria (Table 2B).

To compensate for depletion of Ca⁺⁺ in the cytoplasm, *Gnaq* is expressed in a constant levels (Fig. 3A and 3B) to maintain Ca⁺⁺ concentration in the cytoplasm. In turn, activation of *Calm1* and downstream genes such as *Mlck* and *Camk* to regulate muscle contraction, cell proliferation, and cell apoptosis. Thus, large numbers of genes in the calcium signaling pathway are dynamically regulated in atherogenesis to modulate cell injury and survival. In human,

Mlck (Mylk) has been shown to be associated with early onset coronary artery disease^{33, 34}. Mlck plays an essential role in regulating the influx of Ca⁺⁺ across the plasma membrane in endothelial cells, which regulates endothelial cell retraction³⁵. In our study, the discordance between gene expression and protein expression levels of Mlck suggested that Mlck protein might be degraded very easily, or it is possible that a factor could inhibit the protein expression of Mlck. The markedly decreased of Mlck protein also suggested the decreased control of contractile capacity of endothelial cells. A further study in this direction is warranted.

In our previous study, we show that hepatic calcium signaling in LDb mice was perturbed after high fat diet treatment⁹. The hepatic genes associated with calcium signaling were not the same as the aortic genes of this study. These two studies were designed very differently; the hepatic study compared LDb mice only, whereas the current study compared C57BL/6 control mice to LDb mice. However, both studies suggested disarray of the calcium signaling pathway plays an important role in atherosclerotic lesions development. Recinos et al.³⁶ examined liver gene expression associated with diet and lesion development in *Ldlr*^{-/-} mice. They suggested that the complement pathway was perturbed. Complement cascade was the 2nd pathway identified in our study. A recent work by Collins et al.³⁷ demonstrated that aging accelerated atherosclerosis as the result of increased gene expressions associated with oxidative stress in *Ldlr*^{-/-} mice. Taken together, the diversity of genes associated with atherosclerosis in different animal models indicates the complexity of atherosclerosis development.

Our study of global gene expression in aorta using the atherosclerosis susceptible LDb mouse model identified the calcium-signaling pathway as the most significant pathway that contributes to atherosclerotic lesion development. This study suggested that risk factors of shear stress, genotype (hyperlipidemia), and diet (high-fat), except age influenced gene expression that may alter calcium signaling resulting in progression of lesion development. The next step will be experiments to manipulate expression of genes in the calcium-signaling pathway to investigate their effects on prevention of atherosclerosis.

5. Conclusion

Using a mouse model lacking both *Ldlr* and *Apobec1* genes, we identified 30 out of 185 calcium signaling genes in the KEGG database that were significantly and differentially expressed in this mouse model. Of particular interest is how these risk factors alter the expression plasma membrane receptors and channels that control intracellular cellular calcium flux and signaling, and how are these changes impact cellular and biological processes resulting in pathological development of atherosclerosis. Whether calcium pathways are primarily responsible for the increase in atherosclerosis susceptibility remains to be elucidated. This work suggests further investigation of the biological role of calcium signaling genes in the initiation and progression of cardiovascular disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1A

Figure 1.

Fig. 1. (A). Flow chart for the study design. C57BL/6 (n=16) and LDLb (n=16) mice of 1-month of age were subjected to either a chow or a Western high-fat diet for one month and 7-months. At the designated time, lesion-prone (LP) and lesion-resistant aortic segments (LR) were collected from each animal for RNA extraction and microarray analysis.

Fig. 1(B). Oil-Red O staining of aorta from C57BL/6 and LDLb mice at 8-months of age. Representative Oil-Red O stained aorta from C57BL/6 (1) and LDLb (2) mice are illustrated. The locations of lesion-prone (LP) and lesion-resistant (LR) segments are indicated.

Fig. 1(C). Distribution of cholesterol in plasma separated by FPLC from a male human patient with hyperlipidemia, and for male LDLb and C57BL/7 mice. Pooled plasma samples (200 µl) from 4–6 animals and a patient with hyperlipidemia were fractionated by FPLC. Total cholesterol from each fraction were measured and expressed as mg/dl. The elution fraction for each lipoprotein class is shown.

Fig. 2. Morphology of cultured primary endothelial cells of lesion-prone and lesion-resistant aortic segments from LDb mice

Endothelial cells were cultured from lesion-prone (LP) and lesion-resistant (LR) aortic segments from 2 LDb mice (#1703 and 254). The aortic segments were placed on Matrigel gel until day 7. The morphology of endothelial cells was captured by phase contrast photomicrographs at 10× magnification.

Fig. 3A

Fig. 3B

Figure 3.

Fig. 3A. Western blot analysis of genes associated with calcium signaling pathway in aortic tissues obtained from C57BL/6 and LDb mice

Selected proteins associated with calcium signaling pathway were analyzed by Western blot in aortic tissues obtained from C57BL/6 and LDb mice at 2-months and 8-months of ages fed a chow diet. Aortic homogenates (15 µg) were separated by SDS/PAGE. Protein abundances of Gnaq, Calm1, Atp2a2 (SERCA), Ptger3 (EP3), and Mlck (Mylk) were analyzed and quantified using an Odyssey infrared imaging system (LI-COR). Gapdh was used as control to demonstrate equal sample loading to each lane. Each protein was normalized with the corresponding Gapdh value. Statistical analysis was performed as described in the Methods. The symbol of * represents significant difference of $p < 0.05$. The ratios of LDb/C57BL6 of 2-month and 8-month of each protein are shown.

Fig. 3B. Western blot analysis of genes associated with calcium signaling pathway in primary aortic endothelial cells obtained from C57BL/6 and LDb mice

Aortic endothelial cell protein (15 µg) of LP and LR from C57BL/6 and LDb mice were separated by SDS/PAGE. Protein abundances of Gnaq, Calm1, Atp2a2 (SERCA), Ptger3 (EP3), and Mlck (Mylk) were analyzed and quantified using an Odyssey infrared imaging system (LI-COR). Gapdh was used as control to demonstrate equal sample loading to each lane. . Each protein was normalized with the corresponding Gapdh value. Statistical analysis was performed as described in the Methods. The symbol of * represents significant difference of $p < 0.05$. The ratios of LDb/C57BL6 of LP and LR of each protein are shown.

Fig. 4A

Fig. 4B

Figure 4.

Fig. 4A. The expression of CD11b and CD68 in atherosclerotic lesions in LDb mice. Immunofluorescence staining of aortic sinus sections isolated from C57BL/6 and LDb mice of 8-months of age fed a chow diet were immunostained with anti-CD11b or anti-CD68. The

images were captured using a Zeiss Axio observer.D1m fluorescence microscopy with DAPI, FITC and Texas Red filters.

Fig. 4B. The expression of Gnaq, Calm1, Atp2a2, Ptger3, and Mlck in atherosclerotic lesions in LDb mice

Immunofluorescence staining of aortic sinus sections isolated from C57BL/6 and LDb mice of 8-months of age fed a chow diet were immunostained with anti-Gnaq, anti-Calm1, anti-Atp2a2, anti-Ptger3, or anti-Mlck. The images were captured using a Zeiss Axio observer.D1m fluorescence microscopy with DAPI and Texas Red filters.

Table 1A
Levels of body weight, plasma cholesterol and triacylglycerol, and ratio of HDL cholesterol to plasma cholesterol in wild-type C57BL/6 and LDb (*Ldlr*^{-/-}*ApoBec1*^{-/-}) mice after feeding on a chow diet or a Western High-fat diet

Each value represents the average fasting plasma concentrations of 6–10 mice at the indicated time of diet after one-month of age. Results are presented as means ± S.D. and were analyzed using un-paired student t-test as described in the Materials and Methods section. P values are shown, with p < 0.05 are significant. NS= not significant

Time on diet (months)		1	4	7	1	4	7	1	4	7	1	4	7
Age (months)		2	5	8	2	5	8	2	5	8	2	5	8
		Body weight (g)			TC (mg/dl)			HDL-C/TC			TG (mg/dl)		
Chow													
C57		26±2.0	35±1.7	36±2.8	82±17	81±22	84±20	1.0±0.12	1.41±0.18	1.26±0.15	51±16	45±15	38±5.8
LD <i>b</i>		23±2.5	28±3.2	37±4.3	352±3.1	325±56	334±78	0.17±0.03	0.23±0.05	0.23±0.03	163±37	145±36	148±63
<i>p</i>		NS	NS	NS	1.63E-10	1.30E-08	5.55E-07	5.03E-02	4.03E-02	2.06E-02	8.24E-07	1.91E-06	1.72E-04
High-fat													
C57		22±1.7	34±5.5	46±3.3	69±8.1	215±21	333±57	1.0±0.14	1.1±0.16	0.63±0.04	25±6.0	48±9.5	66±11
LD <i>b</i>		23±1.6	33±5.2	41±9.2	383±95	1278±231	826±193	0.14±0.02	0.30±0.02	0.25±0.04	133±30	514±91	321±73
<i>p</i>		NS	NS	NS	1.01E-06	5.67E-08	4.53E-06	4.21E-02	2.50E-07	4.67E-01	2.85E-07	2.26E-08	2.55E-07

Atherosclerotic lesions in wild-type C57BL/6 and LDb (*Ldlr*^{-/-}*Apobec1*^{-/-}) mice after feeding on a chow diet or a Western High-fat diet
Results are presented as means ± S.D. for the indicated numbers of each genotype. N values are shown in parentheses. Percentage lesions, total atherosclerotic lesion area in mm²/total surface area of the aorta in mm².

Time on the diet (months)		1	7
Age of the animals		2	8
Chow			
<i>C57BL/6</i>		0.54±0.12 (3)	0.18±0.10 (3)
<i>LDb</i>		0.15±0.10 (3)	21±6.5 (6)
<i>p</i>		ns	2.2E-05
High-fat			
<i>C57BL/6</i>		0.06±0.02 (3)	0.10±0.10 (3)
<i>LDb</i>		0.17±0.10 (3)	30±7.2 (6)
<i>p</i>		ns	3.8E-06

Table 2A

Canonical pathway analysis of genes differentially expressed in aorta analyzed from the comparison of genotype, age, diet, and shear stress

The differentially expressed genes were analyzed using Pathway express software to identify underlying biological pathways as described in the Materials and Methods section.

ns= not significantly

Over-represented KEGG pathways	Sum of rank scores	Number of genes expressed in the pathway	P-value	Age	Diet	Shear Stress
Calcium Signaling Pathway	0.818	30	0.0021	ns	0.0002	0.00064
Complement and Coagulation cascades	0.682	17	0.017	0.00021	0.0011	0.02
Focal adhesion	0.676	26	0.0028	0.0063	0.074	ns
Phosphatidylinositol signaling system	0.643	12	0.022	0.011	ns	0.00083
Cytokine-cytokine receptor interaction	0.530	44	ns	3.1E-13	0.0013	ns
MAPK signaling pathway	0.410	19	0.031	ns	0.036	0.025

Table 2B

The fold-changes of differentially expressed genes in the calcium signaling pathways from comparison of genotype, diet, age, and shear stress

The value of fold-change in each comparison was obtained by taking the average normalized expression signal in the treatment group divided by the average expression signal in the control group. The ↑ represents up-regulation and ↓ represents down-regulation. The p values were obtained as described in the Methods

Gene Symbol	Gene Name	Affymetrix ID	Comparison	Fold-changes	P values
Calcium signaling pathway (30 unique genes)					
Adora2b	Adenosin A2b receptor	1434431_x_at	Age	↑1.78	1.30E-03
Atp2a2 (SERCA)	ATPase, Calcium transporting, cardiac muscle, slow twitch 2channel	1416551_at	Stress	↑2.46	4.00E-04
			Diet	↓2.35	5.20E-03
Atp2b1	ATPase, Calcium transporting, plasma membrane 1	1428937_at	Genotype	↑1.83	3.30E-03
Atp2b2	ATPase, Calcium transporting, plasma membrane 2	1420402_at	Stress	↓1.21	1.27E-02
Cacna1c	Calcium channel, voltage dependent, L type, alpha 1C subunit	1421297_a_at	Stress	↑1.73	7.00E-04
Cacna1d	Calcium channel, voltage dependent, L type, alpha 1D subunit	1427974_s_at	Genotype	↓1.79	2.25E-02
Camk4	Calcium/calmodulin-dependent protein kinase	1438960_at	Diet	↑1.55	4.30E-03
Cd38	CD38 antigen	1433741_at	Genotype	↑5.35	1.08E-04
			Age	↑1.58	1.00E-04
Cdc2a	Cell division cycle 2 homolog A	1448314_at	Age	↑1.56	1.00E-03
Gnaq	Guanine nucleotide binding protein	1447593_x_at	Genotype	↓1.52	3.53E-02
			Age	↑1.56	7.60E-03
Htr2b	5-hydroxytryptamine (serotonin receptor 2B)	1422125_at	Genotype	↑1.64	1.50E-03
			Age	↑2.39	1.50E-03
Htr2c	5-hydroxytryptamine (serotonin receptor 2C)	1435513_at	Genotype	↓1.62	2.85E-02
Mlck (Mylk)	myosin, light polypeptide kinase	1439101_at	Stress	↑3.86	2.00E-04
			Diet	↓4.20	2.80E-03
			Genotype	↑8.34	8.00E-04
P2rx1	purinergic receptor P2X, ligand-gated ion channel, 1	1460719_a_at	Stress	↓1.49	4.60E-03
Plcb4	phospholipase C, beta 4	1441531_at	Genotype	↓6.74	5.00E-04
Pln	phospholamban	1450952_at	Stress	↑6.59	7.00E-05
			Age	↑8.46	6.78E-03
Ppp1r12a	protein phosphatase 1, regulatory (inhibitor) subunit 12A	1444762_at	Genotype	↓3.48	1.00E-05

Gene Symbol	Gene Name	Affymetrix ID	Comparison	Fold-changes	P values
PPP3ca	protein phosphatase 3, catalytic subunit, alpha isoform	1426401_at	Genotype	↑1.75	3.30E-03
PPP3cb	protein phosphatase 3, catalytic subunit, beta isoform	1459378_at	Diet	↑1.50	4.37E-02
PRKCB1	protein kinase C, beta	1460419_a_at	Age	↑1.96	1.00E-03
PTGER1	prostaglandin E receptor 1 (subtype EP1)	1445445_s_at	Stress	↓1.24	4.84E-02
PTGER3 (EP3)	Prostaglandin E receptor 3 (subtype EP3)	1450344_a_at	Stress	↓2.45	1.00E-05
PTGER4	prostaglandin E receptor 4 (subtype EP4)	1421073_a_at	Stress	↑1.50	2.40E-03
PTGFR	Prostaglandin F receptor	1420349_at	Stress	↑1.25	2.54E-02
			Diet	↓2.48	1.40E-03
PYGL	liver glycogen phosphorylase	1417741_at	Stress	↓1.84	1.50E-03
RYR2	Ryanodine receptor 2, cardiac	1450123_at	Diet	↓6.23	9.33E-03
SLC25a4	solute carrier family 25 (mitochondrial carrier, adenine nucleotide translocator), member 4	1455069_x_at	Genotype	↑2.45	9.70E-03
SLC8a1	solute carrier family 8 (sodium/calcium exchanger), member 1	1445481_at	Genotype	↓2.85	1.00E-04
SYK	spleen tyrosine kinase	1418261_at	Age	↑1.95	4.80E-03
VDAC1	Voltage-dependent anion channel 1	1415998_at	Genotype	↑2.75	1.85E-04
			Diet	↓2.58	1.40E-03

Table 3

Differentially expressed genes in calcium signaling pathway in primary endothelium from lesion prone and lesion resistant aorta of C57BL/6 and LDb mice

The value of LP/LR from C57BL/6 mice represents average gene expression of LP=3 divided of LR=3 from each gene. The value of LP/LR from LDb mice represents average gene expression of LP=6 divided of LR=7 from each gene LP = lesion prone; LR = lesion resistant; ↑ = up-regulation; ↓ = down-regulation

Gene Symbol	Gene Name	C57BL/6	LDb	During Atherosclerosis	Location
Receptor:		LP/LR	LP/LR	LDb/C57BL/6	
Egfr	Epidermal growth factor receptor	2.26	0.80	↓2.83	Plasma membrane
Htr2b	5-hydroxytryptamine (serotonin receptor 2b)	2.06	2.01	no change	Plasma membrane/cytoplasm
Ptger3	Prostaglandin E receptor 3	1.60	0.44	↓3.64	Plasma membrane
Ptgr	Prostaglandin F receptor	3.03	1.53	↓1.98	Plasma membrane
Ryr2	Ryanodine receptor 2 (component of a calcium channel)	2.06	1.14	↓1.81	Plasma membrane/Nucleus
Tbxa2r	Thromboxane A2 receptor	0.58	2.23	↑3.85	
Calcium Channel:					
Atp2a2 (SERCA)	ATPase, Calcium transporting channel	1.61	1.66	no change	Endoplasmic reticulum (ER)
Cacna1c	Calcium channel, voltage dependent, L type, alpha 1 c subunit	8.06	1.52	↓5.30	Plasma membrane
Slc8a1	Solute carrier protein	1.91	1.16	↓1.65	mitochondria
Vdac1	Voltage-dependent anion channel 1	0.56	1.60	↑2.86	mitochondria
G-protein:					
Gnaq	Guanine nucleotide binding protein	0.80	1.43	↑1.79	Plasma membrane
Kinase protein:					
Calm1	Calmodulin 1, phosphorylase kinase	0.84	1.68	↑2.0	Cytoplasm/nucleus
Camk2d	Calcium/calmodulin-dependent protein kinase	0.85	1.39	↑1.64	Cytoplasm/nucleus
Ip3r1	Inositol 1,4,5-trisphosphate receptor, type 1	1.57	0.71	↓2.21	ER
Mlck (Mylk)	myosin light chain kinase	0.58	2.15	↑3.71	Cytoplasm
Plcb4	phospholipase C, beta 4	1.43	0.97	↓1.47	Cytoplasm
Pln	phospholamban	0.56	1.60	↑2.86	Plasma membrane