This is the peer reviewed version of the following article:

which has been published in final form at:

Type 1 diabetes increases retention of low-density lipoprotein in the atherosclerosis-prone area of the murine aorta

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Word count: 4367   Abstract word count: 238   Figures: 5

ABSTRACT

Background and aims — Individuals with Type 1 diabetes mellitus are at high risk of developing atherosclerotic cardiovascular disease but the underlying mechanisms by which Type 1 diabetes accelerates atherosclerosis remain unknown. Increased retention of low-density lipoprotein (LDL) in atherosclerosis-prone sites of the diabetic vascular wall has been suggested but direct evidence is lacking. In the present study, we therefore investigated whether retention of LDL is increased in atherosclerotic-prone areas using a murine model of Type 1 diabetes.

Methods — Fluorescently-labeled human LDL from healthy non-diabetic individuals was injected into diabetic Ins2[Akita] mice and in non-diabetic, wildtype littermates. The amount of retained LDL after 24 hours was quantified by fluorescence microscopy of cryosections and by scans of *en face* preparations. Vascular gene expression in the inner curvature of the aortic arch was analyzed by microarray and quantitative polymerase chain reaction.

Results — LDL retention was readily detected in atherosclerosis-prone areas of the aortic arch being located in both intimal and medial layers. Quantitative microscopy revealed 8.1-fold more retained LDL in Type 1 diabetic mice compared to wildtype mice. These findings were confirmed in independent experiments using near-infrared scannings of *en face* preparations of the aorta. Diabetic status did not affect arterial expression of genes known to be involved in LDL retention.

Conclusion — Type 1 diabetes increases the ability of the vascular wall to retain LDL in mice. These changes could contribute to the increased atherosclerotic burden seen in Type 1 diabetic patients.

1. Background

Individuals with Type 1 diabetes mellitus are at high risk of developing atherosclerotic cardiovascular disease (ASCVD) independently of traditional CVD risk factors, and the majority of diabetic patients eventually die from ASCVD.[1] Type 1 diabetes infers many metabolic changes that may influence atherogenesis, but the exact mechanisms by which Type 1 diabetes accelerates atherosclerosis remain unknown.[2] Most importantly, while glucose control with anti-diabetic drugs today are efficient in lowering the incidence of microvascular complications, they are much less effective in retarding macrovascular complications, including atherosclerosis.[3]

The arterial wall of Type 1 diabetics is characterized by intimal thickening, functional modifications of the endothelium, increased expression of extracellular proteoglycans, smooth muscle cell proliferation and accumulation of glycated proteins.[4-7] It is widely accepted that a key step in early atherogenesis is retention of apolipoprotein B (apoB)-containing lipoproteins, predominantly low-density lipoprotein (LDL), in atherosclerosis-prone areas of the arterial tree (i.e. the response-to-retention hypothesis).[8] LDL retention is believed to be mediated initially by binding to glycosaminoglycan side chains of proteoglycans within the extracellular matrix.[9, 10] Several types of these are found to be upregulated in Type 1 diabetes,[11-14] indicating that increased LDL retention in pre-lesional arteries in Type 1 diabetes may be a mechanism by which Type 1 diabetes increases atherosclerosis.

However, only few studies have investigated LDL retention in Type 1 diabetes.[12, 15, 16] Mangat *et al.* found in an *ex vivo* model of Type 1 diabetes that retention of remnant lipoproteins in the common carotid artery was increased compared to controls.[12] However, straight segments exposed to laminar blood flow, such as the common carotid artery are protected from plaque development and may therefore not be relevant as a test bed to study the causal mechanisms of diabetes-accelerated lesion progression. In contrast, Proctor *et al.* reported that retention of LDL in the thoracic aorta 2 hours after infusion of LDL was not affected by hyperglycemia.[15] To date, no one have investigated the impact of diabetes on LDL retention in natural atherosclerosis-prone areas in a model in which Type 1 diabetes has been shown to accelerate atherosclerosis.

Others and we have previously shown that Akita mice have increased atherosclerosis development when subjected to hypercholesterolemia.[17, 18] In the present study, we provide evidence for a mechanism that may contribute to this trait by showing that diabetes without concomitant hypercholesterolemia increases the ability of atherosclerosis-prone areas to retain LDL.

2. Materials and methods

An expanded Materials and Methods section is available in the Supplemental Materials and Methods.

2.1 Animals

Type 1 diabetic mice with a mutation in the *Ins2* gene (C57BL/6-Ins2Akita/J; heterozygous) and C57BL/6J mice were obtained from The Jackson Laboratory, Bar Harbor, ME and bred at Aarhus University to generate heterozygous Ins2Akita/J (Akita) and wild-type (WT) littermate controls. Mice were genotyped for the Akita mutation (which abolished a Fnu4HI site) by restriction enzyme digestion of a PCR amplicon from the Ins2 gene (Primerset: FW- 5'TGC TGA TGC CCT GGC CTG CT3' and RV-5'TGG TCC CAC ATA TGC ACA TG3').

All mice enrolled in the experiments were 25-27 weeks old males. Before sacrifice, mice were fasted for 6 hours and blood glucose was measured using a glucometer (CONTOUR®, Bayer Consumer Care AG, Basel, Switzerland). When blood glucose levels exceeded maximum measurable value the maximum value (600 mg/dl) was used.

Mice were fed normal chow and given access to food and water ad libitum. All procedures were approved by the Danish Animal Experiments Inspectorate.

2.2 Purification, labeling and infusion of human LDL

Preparation and characterization of fluorescent LDL was as previously described.[19] Briefly, EDTA-plasma from a healthy, non-fasting individual with a LDL particle count of 1327 nmol/l

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and a Lp(a) level of 307 mg/dl was obtained, and lipoproteins were fractionated by ultracentrifugation at 256,000 g for 20 hours by loading plasma onto a KBr density gradient column (density layers: 1.006, 1.019, 1.063 and 1.21 g/ml). LDL was collected from the 1.063 g/ml density layer and desalted on a PD10 column with PBS before conjugation to Atto 565 or Atto 680 NHS ester fluorochrome (Sigma Aldrich) at pH 8.3. The purity of the LDL preparation was tested by HPLC, which showed only a small contamination of albumin (<1%) (data not shown). Labeled LDL was purified on a PD10 column with PBS. At 24 hours before the mice were sacrificed, labeled LDL corresponding to 500 µg protein per mouse was injected through the tail vein.

2.3 Metabolism of LDL

To compare clearance of LDL in Akita (n=4) and WT (n=8) mice, Atto 565 labeled human LDL (500 µg protein per mouse) was injected into the tale vein. Blood samples were taken 0, 3, 6, 12 and 24 hours post injection, and human apoB-100 in plasma was measured using an ELISA (IBL International) with no cross-reactivity to mouse apoB-100.

2.4 Tissue processing and analysis

For fluorescence microscopy quantification of Atto 565 labeled LDL, the aortic arch, descending aorta, abdominal aorta and the right common iliac artery were harvested. Tissues were cryoprotected, snap-frozen and sectioned at 4 µm thickness. To measure retained LDL in the aortic arch using fluorescence microscopy, sections were analyzed for the presence of Atto 565 signal in an Olympus Cell-R widefield microscope system. LDL was quantified in the center of the inner curvature of the aortic arch in 3 sections taking 40µm apart, using ImageJ version 1.48v (http://rsbweb.nih.gov/ij/). The final intensity was calculated as the mean of intensities on the 3 images. Pixel values from the green channel were subtracted from the red channel (Atto 565), which enabled exclusion of non-specific fluorescence from elastic laminae. For quantification of Atto 680 labeled LDL, *en face* preparations of thoracic aortas were scanned using the 700 nm channel of a LiCor Odyssey Infrared Imaging System.

Endothelial cells were identified by staining with a polyclonal rabbit anti-human von Willebrand Factor (vWF) antibody (A0082, Dako), followed by staining with a FITC goat anti-rabbit secondary antibody (Jackson ImmunoResearch).

2.5 Microarray

Gene expression analysis (RNA) was performed on a small piece of tissue (Ins2Akita n=6; WT n=6) of the inner curvature of the aortic arch (between the branchiocephalic- and left subclavian artery). Total RNA was purified using RNeasy Micro Kit following the manufacturer's instructions (Qiagen). The samples were labeled and hybridized to the Affymetrix GeneChip® Mouse Gene 2.0 ST Array (Santa Clara, CA) covering 35,240 RefSeq transcripts, according to the manufacturer's protocol. All samples were run independently and labeled and scanned in a randomized order to avoid batch effects. Data were processed by the IterPLIER algorithm and analyzed using *R. Student's t-tests.* 4 pre-defined overrepresentation analyses (ORA) (Supplemental Table 2)[19] were performed using GO-Elite8 (http://www.genmapp.org/go_elite/) with default settings and all tested genes as denominator file.

2.6 Real-time PCR

Quantitative polymerase chain reaction (QPCR) was used to confirm a change in proteoglycan 4 (*Prg4*) gene expression (*Prg4*: F- 5'AGTTTGGGGTATTCCCTCTCC3' and R- 5'TGAATGTTGCCACCTCTCTTGA3'). A Brilliant III Ultra Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies) was used on a Stratagene Mx3005P (Agilent Technologies) with the MxPro QPCR Software (Agilent Technologies). Conditions for the reaction were as follows: 10 minutes at 50°C and 3 minutes at 95°C followed by 40 cycles of 20 seconds 95°C and 20 seconds at 60°C. In the first analysis the same tissues that had been used for the microarray were used. In the second analysis, we re-tested for *Prg4* gene expression in a new groups of mice (Ins2Akita n=8; WT n=6). Expression levels were estimated and normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level.

2.7 Statistical Analysis

All statistical analysis were performed using the Prism statistical software (GraphPad, San Diego, CA). Prior to analysis data was tested for normal distribution. Blood glucose and weight followed a normal distribution and were compared between groups using Student's t-tests. In other cases, two-sample nonparametric comparisons were performed using *Mann-Whitney test*. Comparisons of LDL clearance curves between groups were done by *repeated measures two-way ANOVA*. $p<0.05$ was considered to be statistically significant.

3. Results

3.1 Characteristics of mice

Fasting glucose and final weight of Akita and WT mice used in the study are shown in Figure 1. Akita mice had a significant lower body weight compared with WT mice (25.6 ± 0.5 g and 31.9 ± 0.3 g, respectively; $p<0.05$) while fasting plasma glucose was significantly elevated (550.0 ± 14.7 mg/dl and 194.5 ± 8.2 mg/dl, respectively; $p<0.05$) (Fig. 1). Mean values of weight and fasting plasma glucose of mice from each of the 4 independent experiments carried out are provided in Supplemental data (Supplemental Table 1).

3.2 Retention of LDL in the arterial wall

To determine the location of retained LDL in the arterial wall, Atto 565-labeled human LDL was injected intravenously into Akita and WT mice, and arteries were analyzed 24 hours later for the presence of fluorescent LDL (Fig. 2). At that time point, injected LDL was completely cleared from the circulation (Fig. 3) and LDL observed in the arterial was thus interpreted as being retained. We confirmed the labeling technique by immunofluorescence staining with an anti-human apoB antibody (with no cross-reaction to murine apoB), which was found to bind in the areas positive for Atto 565 (Supplemental Fig. 1).

In the atherosclerosis-prone inner curvature of the aortic arch, LDL retention was primarily seen in the intimal layer (Fig. 2A-D) and to a lesser degree in the medial layer in both groups (Fig. 2D). LDL was never observed in the medial layer alone without also being retained in the overlying intima. No retention of LDL was seen in the outer curvature of the aortic arch, and, except for a few cases of LDL retention near aortic branch points, neither in the descending aorta or iliaca (data not shown). To determine the exact localization of LDL retention in the intima, sections with Atto 565 labeled LDL were stained for endothelial cells (vWF) using a green fluorescent secondary antibody. Labeled LDL appeared to be located within endothelial cells as well as in the subendothelial space (Fig. 4).

To assess if Type 1 diabetes increases the ability of the arterial wall to retain LDL, retention of LDL in Akita and WT mice was quantified by analyzing Atto 565 fluorescence in the inner curvature of the aortic arch. Substantially more LDL was found retained in Akita (n=9) compared to WT mice (n=6) with a fold difference of 8.1 (Fig. 2E).

To verify these findings, we repeated the experiment, but this time using Atto 680-labeled LDL and quantification of retention in thoracic aortas opened and positioned *en face* with a near infrared imaging scanner. As in the experiment using Atto 565, LDL was located at atherosclerosis prone areas in the aortic arch, primarily in the inner curvature (Fig. 5A). Significantly more LDL was retained in Akita mice (n=7) compared to WT mice (n=8) with a fold difference of 1.89 (Fig. 5B).

3.3 Clearance of LDL

Altered LDL clearance has been reported in Type 1 diabetes.[20] Furthermore, the weight difference between Akita and WT mice and the use of a fixed dose of fluorescent LDL could lead to differences in the concentration of labeled LDL in the circulation. This could potentially bias our analysis of LDL retention by changing the amount of LDL available to the arterial wall. We therefore assessed peak concentration and clearance rate of human LDL in Akita and WT mice. Blood samples were taken at 5 different time points after intravenous injection of human LDL and the concentration of human apoB-100 in plasma was measured. The peak

concentrations of LDL in blood after injection and the plasma clearance rate were similar in the two groups (Fig. 3).

3.4 Gene expression in an atherosclerosis-prone area of the diabetic aortic arch

We used gene-expression profiling by microarray to look for genes involved in the changed ability of the diabetic inner aortic arch to bind LDL. To this end, overrepresentation analyses were performed for predefined 4 gene sets that we recently found to be regulated when the straight segment of the murine carotid artery, which do not retain LDL, is transformed into an LDL-binding, atherosclerosis-prone segment by disturbed laminar flow[19]: 1) proteoglycan core proteins associated with lipoprotein retention. 2) markers of synthetizing vascular smooth muscle cell (VSMC). 3) markers of contractile VSMC and 4) chondroitin sulfate/dermatan sulfate glycosaminoglycan synthesizing and sulfating enzymes. However, none of these pre-defined gene sets were significantly over-represented among genes showing differential expression between diabetic and non-diabetic mice (Supplemental Table 2).

In an explorative analysis of the data, we found that Prg4 was the only upregulated ECM protein encoding mRNA upregulated by more than 1.5 fold in Type 1 diabetes (fold change=1.52; *p*=0.055). *Prg4* has not been investigated in relation to LDL retention or atherosclerosis, but was also found upregulated in our previous experiment in which LDL-binding properties of murine carotid arteries were induced with disturbed laminar blood flow.[19] Immunofluorescence staining showed PRG4 in the arterial wall, mostly intracellularly in smooth muscle cells and to a lesser degree and more variably in endothelial cells (Supplemental Figure 2). We did not find a clear overlap between sites of cellular expression and labeled LDL across sections, but importantly we were not able to detect an extracellular form of PRG4, possibly because of masking of the antibody by GAG-chains. We decided to confirm the upregulation of the *Prg4* gene using qPCR on the same sample set and on a new sample set. Levels of selected genes relative to that of a control gene, GADPH, were compared between an Akita- and WT atherosclerosis prone area of the aortic arch. *Prg4* was again found to be upregulated with a foldchange of 2.13 (*p*=0.059). QPCR analyses of *Prg4*

on a new dataset further confirmed the upregulation of *Prg4*, now being significant (fold change of 1.78; *p*=0.011).

4. Discussion

Atherosclerosis is the principal cause of mortality in people with diabetes but it is unknown whether type 1 diabetes and type 2 diabetes accelerate atherogenesis through similar mechanisms.[1] Type 1 diabetic patients most often have normal plasma lipid levels, although their lipoprotein composition may be abnormal, while type 2 diabetic patients frequently display dyslipidemia and other cardiovascular risk factors.[22] However, dyslipidemia and cardiovascular risk factors seen in type 2 diabetes cannot fully explain the increase in cardiovascular events. Thus, it is becoming increasingly clear that hyperglycemia and suboptimal blood glucose control might result in adverse effects in large blood vessels and thereby accelerate cardiovascular disease and atherosclerosis. The cellular and molecular mechanisms underlying this are, however, still poorly understood.

In the present study we demonstrate that atherosclerosis-prone sites in diabetic Akita mice display increased retention of exogenous LDL compared to non-diabetic control mice. By facilitating LDL retention in the arterial wall, which is a key limiting step in the initiation of atherosclerosis,[21] this may point to one mechanism by which diabetes contributes to increased risk of ASCVD.

Experimental approach

Diabetes accelerates atherosclerosis in a number of hyperlipidemic mouse strains.[23-26] In most of these, however, diabetes also augments plasma cholesterol levels substantially making it difficult to single out the effects of diabetes on the development of atherosclerosis.[23-26] This problem also applies to the Type 1 diabetic Akita mouse model.[17, 18, 27] When backcrossed into *Ldlr* knockout mice[18] or when rendered functional LDL receptor deficiency by AAV-mediated

PCSK9 transfer[17], the level of total cholesterol increases massively biasing any attempt to investigate the isolated effect of diabetes on the atherosclerotic process.

In the present study we circumvented this issue by analyzing normocholesterolemic Akita diabetic mice for changes in the vascular wall that are likely to predispose it for development of atherosclerosis. We performed the study in 26 weeks old mice expecting that longer exposure to hyperglycemia would give time for any (structural) vascular changes in the arteries to fully evolve.[28] We have previously shown that hypercholesterolemia induced by PCSK9 gene transfer at this time point leads to accelerated atherosclerosis in Akita mice compared to normal mice.[17]

LDL retention in diabetes

The "response-to-retention" theory of atherosclerosis has been intensively investigated in non-diabetic mice, and it is now well documented that retention of LDL particles in the extracellular matrix, especially through binding to proteoglycans, is a key limiting step in the initiation of atherosclerosis.[10, 21] Also it is clear that the difference in LDL binding properties across the vascular tree is an important reason for the multifocal topography of atherosclerosis, with the atherosclerosis-prone sites around branch points and in curvatures showing much higher levels of LDL retention than atherosclerosis-resistant segments.[19, 21, 29]

Several observations indicate that LDL retention could be affected by diabetes. First, the diabetic arterial wall is characterized by increased expression of extracellular proteoglycans as well as the accumulation of glycated proteins which may mediate LDL retention.[30] Second, prolonged hyperglycaemia is associated with excessive cross-linking of collagen and ECM proteins in the vascular wall, resulting mainly from glycation of proteins and formation of advanced glycation end products (AGEs), which could lead to both accumulation and subsequent oxidative modification of LDL.[31, 32] Third, changes in LDL retention could lead to changes in the distribution of atherosclerosis. Indeed, diabetic patients tend to develop atherosclerosis that affect the periphery of the arterial tree more than in non-diabetic patients often leading to clinical peripheral arterial disease.[33]

Despite the supporting evidence, experiments directly examining the impact of diabetes on LDL retention are lacking. One study demonstrated an increased retention of chylomicron remnants *ex vivo* in Type 1 diabetic rats in which remnants were shown to co-localize with arterial biglycan and glycated matrix proteins.[12] In other studies, hyperglycemia was shown to promote arterial uptake of triglyceride rich remnant lipoproteins, but not LDL, in rabbits and rats.[15] All of these studies, however, were based on the straight segment of the carotid artery that does not normally develop atherosclerosis. Furthermore, the amount of arterial lipoprotein was determined <2 hours after intravenous injection or the start of *ex vivo* incubation when labeled lipoproteins were still present in the blood or incubation medium. Therefore the amount of labeled LDL in the arterial wall reflected endothelial permeability rather than the ability of the artery to retain (i.e. immobilize) lipoproteins. Importantly, several studies in rabbits and mice have shown that endothelial permeability is not limiting for LDL retention in the arterial wall. In these models, atherosclerosis-prone sites differ consistently from atherosclerosis-resistant sites with respect to capacity for LDL retention, but not always with respect to endothelial permeability.[19, 21, 29]

Retention of LDL has also been examined in Cynomolgus monkeys forty-eight hours after injection.[16] Here, they found an increase in arterial LDL accumulation in the femoral arteries in diabetic animals but not in the aorta. However, as the authors note, the increased LDL accumulation might have reflected the development of more femoral atherosclerosis in the diabetic compared to non-diabetic monkeys. Importantly, whereas these studies provide some insight into lipoprotein handling in the diabetic arterial wall, none of them investigated retention of lipoproteins in natural, nondiseased atherosclerosis-prone areas.

In the present study we found LDL to be retained in both the intimal and medial layers of atherosclerosis-prone sites. This is in agreement with our recent study on lipoprotein retention in the artery wall induced by atherogenic blood flow[19] and has also been found by others.[10, 12] When challenged with Type 1 diabetes substantially more LDL was retained. The increased retention of LDL could not be explained by an altered LDL peak concentration or clearance. The similar peak concentration of LDL in Akita mice compared to WT mice, might be

counterintuitive since the groups differed in weight but was injected with the same dose of labeled LDL. However, the low weight of Akita mice is mainly explained by a lower amount of fat, and the distribution volume for LDL may not be similarly reduced.[34] Also, even though differences in LDL clearance has been reported to exist in Type 1 diabetes,[20] our results are in agreement with at study in streptozotocin-induced diabetic mice in which LDL isolated from control animals were cleared at the same rate in streptozotocin-induced mice and control mice.[20]

In an attempt to gain insight into potential gene expression changes in the artery wall that may underlie the changes in LDL retention, we conducted an overrepresentation analysis testing for regulation of several pre-defined gene sets known to be involved in the control of LDL retention. We did not detect significant enrichment of any of these gene sets, but cannot exclude that this type of regulation is involved. A similarly powered study was able to detect regulation of these gene sets by disturbed blood flow[19], but similar changes could be induced by diabetes at a lower magnitude and still lead to important structural changes over time. It is, however, also possible that genes not yet known to be involved in the regulation of LDL retention is involved. Our gene expression analysis did not have the power to test for regulation of individual genes, but by inspection of data we noticed that *Prg4*, which we have previously shown to be increased in sites of atherogenic blood flow,[19] was also increased by Type 1 diabetes and we confirmed this by RT-PCR in an independent set of arteries. PRG4/lubricin has mostly been studied in joints where it is secreted from chondrocytes into the synovial fluid in both glycoprotein and proteoglycan forms and reduces friction of the cartilage surface.[35] So far, there have been no studies investigating the role of PRG4 in the arterial wall and its relation to retention of lipoproteins, but it is expressed at relevant sites in the arterial wall, and is upregulated in both flow-induced[19] and, as shown here, at sites with diabetes-induced increases in the ability of the arterial wall to bind LDL. Further studies are needed to elucidate whether it is directly involved in LDL retention, but interestingly, PRG4 directly bind Toll-like receptors (TLR2, TLR4, TLR5) and modulate down-stream NF-kB

signaling *in vitro*,[35] indicating another potential pathway by which changes in arterial PRG4 expression may influence atherosclerosis susceptibility.

It remains possible that the cause of increased ability to bind LDL in diabetic mice could be mediated by a circulating bridging molecule or some other mechanism not controlled by local vascular gene expression. Hyperglycaemia can lead to glycation or glycoxidation of lipoproteins, which in turn could affect their retention in the arterial wall by increased proteoglycan binding properties.[36]

Limitations

Because the density range of Lp(a) (1.050–1.12 kg/l) partly overlaps with the density interval that we used to collect LDL (1.019–1.063 kg/l), our LDL preparation may have contained some Lp(a). Lp(a) binds the arterial wall matrix with higher affinity than that of LDL by interacting with a least partly the same type of GAGs.[37] Increased binding of Lp(a) in arteries could both contribute to accelerated atherosclerosis in diabetic humans and to the fluorescent lipoprotein retention signal in the present study. Since the number of Lp(a) particles in our donor plasma was an estimated 13-fold lower than LDLs before the preparative ultracentrifugation, which further facilitates LDL over Lp(a) purification[38], the contribution is expected to be of low magnitude, but it cannot be ruled out.

5. Conclusion

Type 1 diabetes in mice is followed by increased LDL retention at atherosclerosis prone sites. This is a possible link between Type 1 diabetes and increased development of atherosclerosis.

Conflict of interest

None.

Financial support

Author contributions

Mette K. Hagensen – conception and design, acquisition of data, analysis and interpretation of data, drafting the article

Martin Bødtker Mortensen – acquisition of data, critical revision

Mads Kjoelby - acquisition of data, critical revision

Ninna L. Stillits - acquisition of data, critical revision

Lasse B. Steffensen - acquisition of data, critical revision

Jacob F. Bentzon - conception and design, critical revision

Acknowledgments

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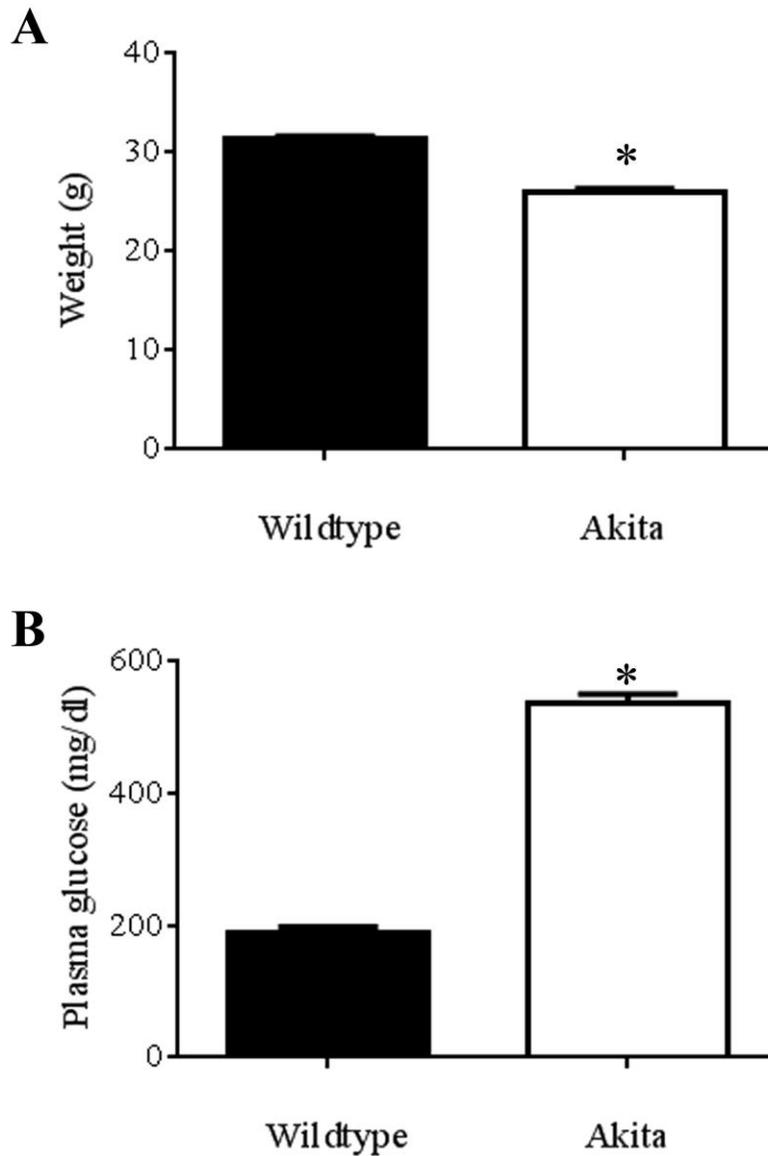
Figures

Figure 1. The Type 1 diabetic phenotype of Akita mice. Akita mice (n=26) showed reduced weight (A) and increased plasma glucose levels (B) compared to wildtype mice (n=27). Plasma glucose was determined by tail blood samples collected before sacrifice after 6 h fasting. Asterisks indicate statistical significance compared to wildtype (p<0.0001; *Student's t-test*). Error bars indicate SEM.
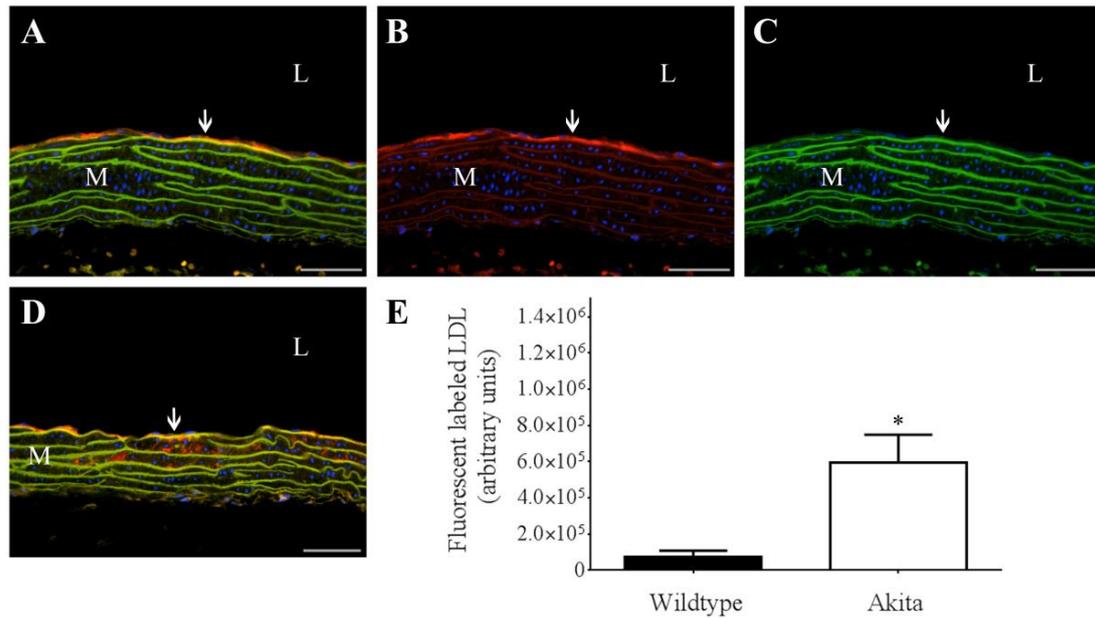
Figure 2. The Type 1 diabetic arterial wall has increased ability to retain low-density lipoprotein (LDL). (A-D) Atto 565-labeled LDL is retained in atherosclerosis prone areas of the aorta 24 hours after infusion. Most of the retained LDL is located in the intimal layer [red in A+B+D] but is also found to be located in the medial layer [red in D]. (E) The amount of LDL retained in the arterial wall was calculated in ImageJ by subtracting the autofluorescence of the elastic laminae in the green channel [C] from the autofluorescence in the red channel [B]. The Type 1 diabetic arterial wall (n=9) has significant more retained LDL compared to the wildtype arterial wall (n=6). Red indicates Atto 565-labeled LDL; green; autofluorescence; blue, nuclei; A+D, merged color channels; B, red channel; C, blue channel; M, media; L, lumen; arrows, endothelium. Asterisks indicate statistical significance compared to wildtype (*p*<0.05; *Mann-Whitney test*). Error bars indicate SEM. Scalebars=50 μm.
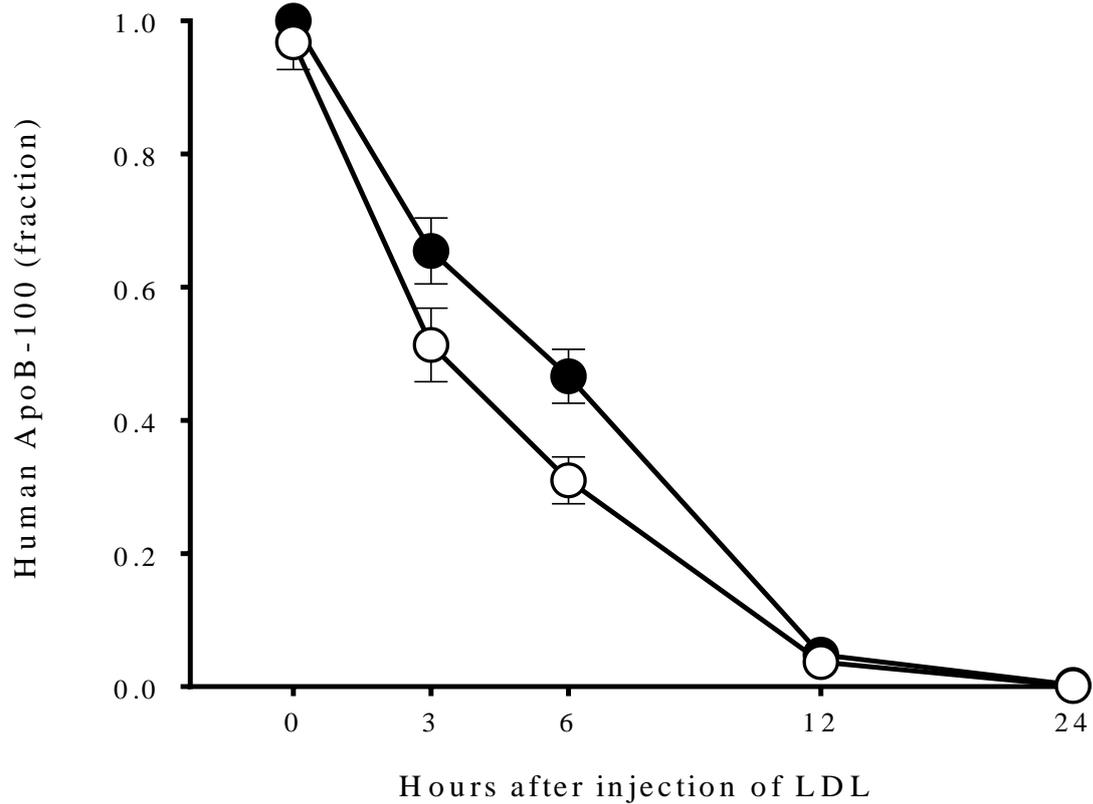
Figure 3. Infused human low-density lipoprotein (LDL) is cleared from the circulation within 24 hours. Clearance of human apoB100 in murine plasma in wildtype mice (closed symbols; n=8) and Akita mice (open symbols; n=4) 1, 3, 6, 12 and 24 hour after LDL infusion as assayed using a human-specific apoB ELISA. Asterisks indicate statistical significance compared to wildtype mice (*p*<0.05; *Student's t-test*). Error bars indicate SEM.
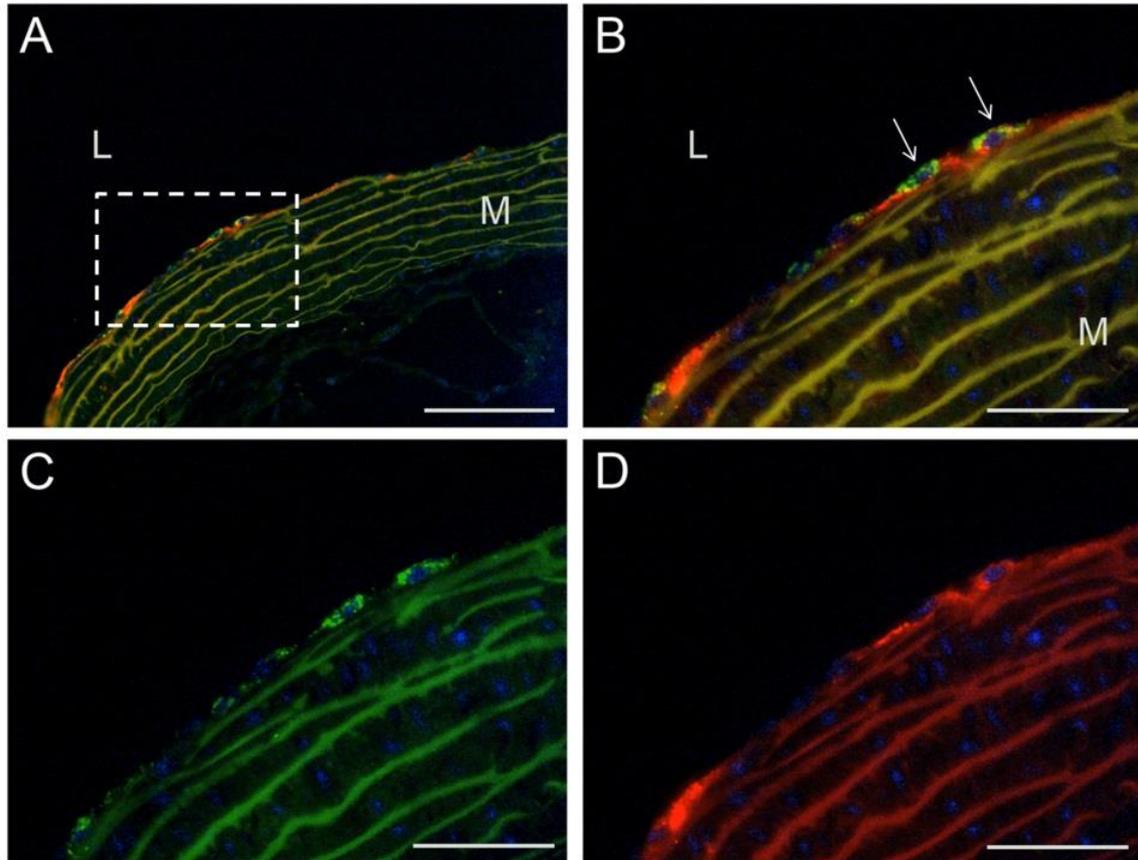
Figure 4. Accumulation of infused low-density lipoprotein (LDL) in endothelial cells. (A) Representative image of the inner curvature of the aortic arch from a Type 1 diabetic mouse infused with Atto 565 labeled LDL (red) and stained for vWF in endothelial cells (green). Scale bar 50 µm. (B-D) Higher magnification of the demarcated area. Scale bars 20 µm. Red indicates Atto 565; green, vWF; blue, DAPI; L, lumen; M, media.
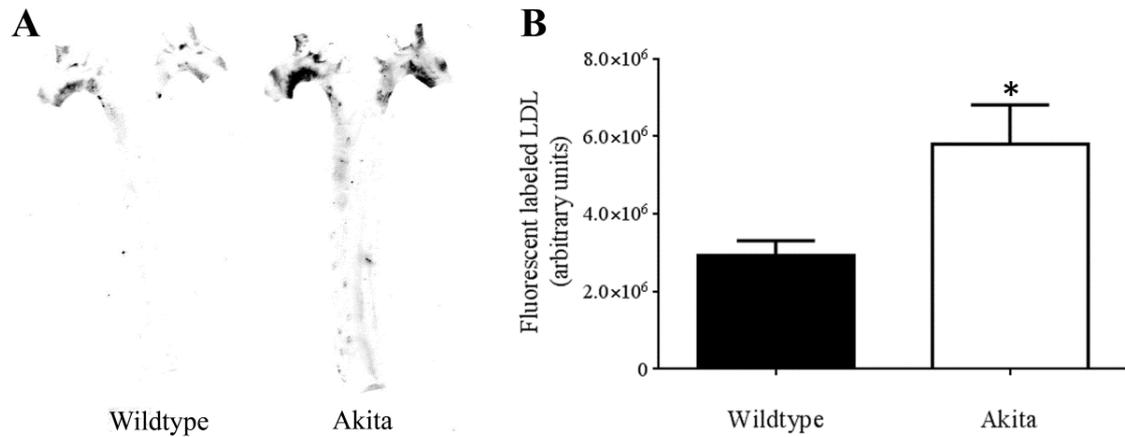
Figure 5. The Type 1 diabetic arterial wall retains more LDL. (A) Representative images of atto 680-labeled LDL retained in longitudinally opened aortas in wildtype and Akita mice 24 hours after infusion. (B) Diabetic aortas (n=7) have increased LDL retention compared to the aorta from wildtype mice (n=8). Asterisks indicate statistical significance compared to wildtype ($p<0.05$; *Mann-Whitney test*). Error bars indicate SEM.

Supplemental Materials and Methods

2.2 Purification, labeling and infusion of human LDL

Preparation and characterization of fluorescent LDL was as previously described.[19] Briefly, blood from a healthy, non-fasting individual was collected into vacutainer tubes containing EDTA and centrifuged at 3,000 g for 10 min at 4°C. LDL was isolated by ultracentrifugation at 256,000 g for 20 hours by loading plasma onto a KBr density gradient column (density layers: 1.006, 1.019, 1.063 and 1.21 g/ml).

After ultracentrifugation, LDL was collected from the 1.063 g/ml density layer and desalted on a PD10 column with PBS before conjugation to Atto 565 or Atto 680 NHS ester fluorochrome (Sigma Aldrich) at pH 8.3. Labeled LDL was purified on a PD10 column with PBS. A sample of isolated LDL from the donor (fluorescently labeled) was analyzed for purity using a biocompatible Dionex HPLC Ultimate 3000 system equipped with a fluorescent detector (Dionex Denmark, Hvidovre, Denmark) and a TSKgel SuperSW3000 column (Tosoh Bioscience GmBH, Stuttgart, Germany). 300 mM KCl, 20 mM Tris pH 7.4 was used as running buffer. At 24 hours before the mice were sacrificed, labeled LDL corresponding to 500 µg protein per mouse was injected through the tail vein.

Lp(a) in plasma of the donor was 307 mg/dl (Unilabs, Copenhagen, Denmark), corresponding to approximately 100 nmol/l,[1] and LDL particle concentration was 1327 nmol/l (LipoScience, Inc., Raleigh, NC). Since the density range of Lp(a) (d 1.050–1.100 kg/l) overlaps with the range used to collect LDL (1.019-1.063 kg/l), some Lp(a) may co-purify with LDL in the preparative ultracentrifugation.

2.4 Tissue processing and analysis

Anesthetized mice (pentobarbital 5 mg i.p.) were perfused with 0.9% NaCl solution and subsequently perfusion-fixed with phosphate-buffered 4% formaldehyde (pH 7.2) through the left ventricle for 1 and 5 minutes, respectively. The mice were further immersion-fixed for 6 hours at room temperature. For fluorescence microscopy quantification of Atto 565 labeled

LDL, the aortic arch, descending aorta, abdominal aorta and the right common iliac artery were harvested. Tissues were cryoprotected in sucrose solution (25 % w/v for 24 h + 50 % w/v for 24 h), embedded in O.C.T™ compound (Sakura Finetek) and snap-frozen in liquid nitrogen-chilled methanol:acetone (1:1), and sectioned longitudinally, except for the right common iliac artery which were cross-sectioned, at 4 µm thickness. To measure retained LDL in the aortic arch using fluorescence microscopy, sections of the aortic arch were stained with DAPI (Sigma), mounted with Slowfade Light Antifade (Invitrogen) and analyzed for the presence of Atto 565 signal in an Olympus Cell-R widefield microscope system using the Xcellence Imaging Software (Olympus). The threshold for quantification of fluorescence signal was set by analyzing sections from 4 mice which had received an injection of saline. LDL was quantified in the center of the inner curvature of the aortic arch in 3 sections taking 40µm apart, using ImageJ version 1.48v (http://rsbweb.nih.gov/ij/). The final intensity was calculated as the mean of intensities on the 3 images. Pixel values from the green channel were subtracted from the red channel (Atto 565), which enabled exclusion of non-specific fluorescence from elastic laminae. For en face quantification of Atto 680 labeled LDL, the thoracic aortas were cleaned for periadventitial tissue, opened longitudinally and mounted on microscope slides using Aquatex (Merck) and coverslips. En face preparations were scanned using the 700 nm channel of a LiCor Odyssey Infrared Imaging System. To determine background levels, mice which had not been injected with LDL were used.

Endothelial cells were identified by staining with a polyclonal rabbit anti-human von Willebrand Factor (vWF) antibody (Dako, A0082; 1:200 dilution) for 1 hour at room temperature and Proteoglycan 4 was detected by staining with anti-human Proteoglycan 4 serum with cross reactivity to murine Proteoglycan 4 (Antibodies online, ABIN347549; 1:500 dilution, overnight). For both antibodies the staining was followed by staining for 1 hour with FITC goat anti-rabbit secondary antibody (Jackson ImmunoResearch; 1:400 dilution). Prior to staining sections were blocked for unspecific binding with normal goat serum (Jackson ImmunoResearch, West Grove, Pa; 10%) for 30 minutes. IgG antibodies from nonimmunized rabbits at the same concentration served as a negative control (rabbit IgG, X0903, Dako). Apolipoprotein B (apoB)

was identified by staining overnight with a goat polyclonal to Apolipoprotein B (Abcam, ab98132; 1:100 dilution) after blocking with normal donkey serum (Jackson ImmunoResearch; 10%) for 30 minutes, followed by staining with a FITC Donkey Anti-Goat (Jackson ImmunoResearch) secondary antibody for 1 hour. Goat serum at the same concentration served as a negative control (X0907, Dako). All of the antibodies were diluted in 1% normal serum in TBS. Cell nuclei were stained with DAPI (Sigma, St Louis, Mo). Sections were mounted in Slowfade Light Antifade (Invitrogen, Carlsbad, Calif). eGFP was detected by its natural fluorescence.

2.5 Microarray

Gene expression analyses (RNA) were performed on a small piece of tissue (Ins2Akita n=6 ; WT n=6) of the inner curvature of the aortic arch (between the branchiocephalic- and left subclavian artery). Briefly, anesthetized mice were flushed with RNAlater RNA Stabilization Reagent (Qiagen) via the left ventricle to immediately stabilize tissues before the tissue was excised and snap frozen in liquid nitrogen. The samples were stored at -80°C until further analysis. Total RNA was purified using RNeasy Micro Kit following the manufacturer's instructions (Qiagen). The RNA quality was tested on an Agilent 2100 Bioanalyzer, and samples with a 28S/18S ratio < 1.0 and RNA integrity number < 7 were excluded. The RNA quality was tested on an Agilent 2100 Bioanalyzer. The samples were labeled and hybridized to the Affymetrix GeneChip® Mouse Gene 2.0 ST Array (Santa Clara, CA) which covers 35,240 RefSeq transcripts, according to the manufacturer's protocol. All samples were run independently and labeled and scanned in a randomized order to avoid batch effects. Data were processed by the IterPLIER algorithm and analyzed using *R. Student's t-tests.* 4 pre-defined overrepresentation analyses (ORA) (Supplemental Table 2) were performed using GO-Elite8 (http://www.genmapp.org/go_elite/) with default settings and all tested genes as denominator file. Genes with differential expression (p-value<0.05 and transcription level difference of >10%) were used as input file and the number of permutations set to 2000. Here, gene sets with "proteoglycan core proteins associated with lipoprotein retention", "markers of

synthetizing vascular smooth muscle cell (VSMC)", "markers of contractile VSMC" and "chondroitin sulfate/dermatan sulfate glycosaminoglycan synthesizing and sulfating enzymes" were tested (Supplemental Table 1).[19] When z scores were above 1.96 gene sets were considered significantly over-represented in the analysis.

Supplemental data

Supplemental Tabel 1. Blood glucose and weight of WT and Type 1 diabetic mice. Asterisks indicate statistical significance compared to wildtype (p<0.05; Student's t-test)

	WT mice	Type 1 diabetic mice
Atto 565LDL retention		
Number	6	9
Blood glucose (mg/dl)	165.8 ± 7.5	591.2 ± 8.8*
Weight (g)	31.6 ± 0.7	25.8 ± 0.6*
LDL clearance		
Number	8	4
Blood glucose (mg/dl)	207.2 ± 9.3	462.6 ± 9.5*
Weight (g)	32.3 ± 0.4	27.4 ± 0.4*
Microarray		
Number	5	6
Blood glucose (mg/dl)	212.2 ± 22.9	546.6 ± 26.9*
Weight (g)	31.5 ± 0.4	24.1 ± 0.9*
Atto 680LDL retention		
Number	8	7
Blood glucose (mg/dl)	172.5 ± 16.4	506.3 ± 18.1*
Weight (g)	30.0 ± 0.6	27.2 ± 0.5*

**Supplemental Table 2.** Predefined Gene Sets analyzed by GO-Elite.

Gene set	Effect of disturbed flow	Genes in gene set
Constrictive VSMC phenotype	Downregulation	Itga1, Myocd, Cald1 (h-caldesmon), Vcl, Actg2, Speg, Myh11, Smtn, Itgb1, Itga7, Cdh13, Cdh2, Acta2, Des, Tagln, Aebp1, Cnn1
Synthesizing VSMC phenotype	Upregulation	Pdgfa, Spp1, Mgp, Gja1, Sdc1, Msn, Rbp1, Myh10, Cald1 (l-caldesmon), Icam1, Sdc4, Col1a1, Mmp9
Proteoglycan core proteins associated with lipoprotein retention	Upregulation	Bgn, Dcn, Vcan, Acan, Lum, Hspg2
CS/DS GAG synthesizing and sulfating enzymes	Upregulation	Xylt1, B3galt6, Csgalnact1, Chsy1, Chpf, Dsel, Chst11, Chst1, Chst15, Xylt2, B4galt7, B3gat3, Csgalnact2, Chsy2, Chsy3, Dse, Ust
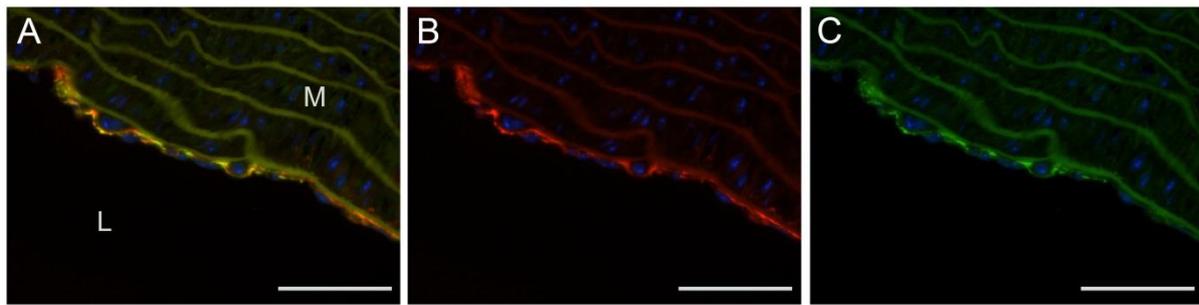
Supplemental Figure 1. Apolipoprotein B co-localize with LDL in the subendothelium.
(A) Representative image of the inner curvature of the aortic arch from a Type 1 diabetic mouse infused with Atto 565 labeled LDL (red in A+B). Apolipoprotein B (green in A+C) is found to co-localize with Atto 565 labeled LDL. Red indicates Atto 565; green, apolipoprotein B; blue, DAPI; L, lumen; M, media. Scale bars 50 μm.
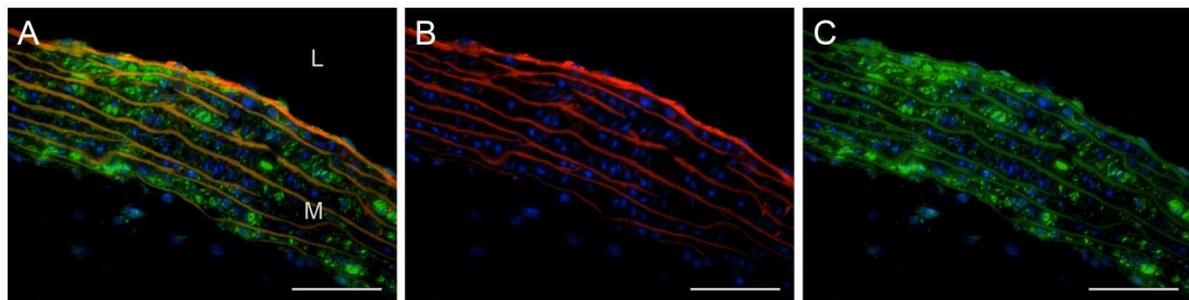
Supplemental Figure 2. Proteoglycan 4 is evenly distributed in the in inner curvature of the aortic arch. (A) Representative image of the inner curvature of the aortic arch from a Type 1 diabetic mouse infused with Atto 565 labeled LDL (red in A+B) and stained for proteoglycan 4 (green in A+C). Proteoglycan 4 is located intracellularly and also in co-localization with Atto 565 labeled LDL. Red indicates Atto 565; green, proteoglycan 4; blue, DAPI; L, lumen; M, media. Scale bars 50 μm.

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