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Hyperglycemia Impairs Atherosclerosis Regression in Mice

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Diabetic patients are known to be more susceptible to atherosclerosis and its associated cardiovascular complications. However, the effects of hyperglycemia on atherosclerosis regression remain unclear. We hypothesized that hyperglycemia impairs atherosclerosis regression by modulating the biological function of lesional macrophages. HypoE (*Apoe*$^{h/h}$Mx1-Cre) mice express low levels of apolipoprotein E (apoE) and develop atherosclerosis when fed a high-fat diet. Atherosclerosis regression occurs in these mice upon plasma lipid lowering induced by a change in diet and the restoration of apoE expression. We examined the morphological characteristics of regressed lesions and assessed the biological function of lesional macrophages isolated with laser-capture microdissection in euglycemic and hyperglycemic HypoE mice. Hyperglycemia induced by streptozotocin treatment impaired lesion size reduction (36% versus 14%) and lipid loss (38% versus 26%) after the reversal of hyperlipidemia. However, decreases in lesional macrophage content and remodeling in both groups of mice were similar. Gene expression analysis revealed that hyperglycemia impaired cholesterol transport by modulating ATP-binding cassette A1, ATP-binding cassette G1, scavenger receptor class B family member (CD36), scavenger receptor class B1, and wound healing pathways in lesional macrophages during atherosclerosis regression. Hyperglycemia impairs both reduction in size and loss of lipids from atherosclerotic lesions upon plasma lipid lowering without significantly affecting the remodeling of the vascular wall. *(Am J Pathol 2013, 183: 1981–1992; http://dx.doi.org/10.1016/j.ajpath.2013.08.019)*

Evidence supporting the regression of atherosclerotic lesions as a viable therapeutic goal has increased considerably since the first studies reporting the shrinkage of vascular lesions in both animal models and clinical trials. A drastic and sustained lowering of plasma cholesterol levels is required to induce regression of atherosclerotic lesions.[1] Mechanisms identified in atherosclerosis regression include decrease in lipoprotein retention within the vascular wall,[2,3] increase in reverse cholesterol transport from foam cells,[2,4–6] emigration, reduced vascular recruitment of inflammatory macrophages,[1,7] and increased recruitment and conversion of macrophages to a phenotype favoring the healing process of the vessel wall.[2,3,8,9] Nevertheless, despite progress made in controlling hyperlipidemia, clinical therapeutic achievement of atherosclerosis regression remains limited. Unfortunately, most patients treated for dyslipidemia neither reach nor sustain plasma lipid-lowering levels required to achieve atherosclerosis regression.[10] Moreover, although shrinkage of lesion volume is desirable, the remodeling and stabilization of vulnerable plaques is required to reduce potential adverse cardiovascular outcomes.[1] Hence, a greater understanding of the mechanisms of atherosclerosis regression is needed to help generate effective therapeutic treatments.

Treatment of atherosclerosis is particularly challenging for individuals suffering from obesity and diabetes. Diabetics are more susceptible to atherosclerosis and its associated cardiovascular complications.[11,12] Although intensive hyperglycemia management reduces cardiovascular events among type 1 diabetic patients, conflicting results have emerged from studies performed among type 2 diabetic patients.[13] In addition, a clear distinction between the effects of hyperglycemia and those of hyperlipidemia on the development of atherosclerosis has been difficult to establish.[14] Thus, whether hyperglycemia can modulate atherosclerosis regression remains unclear.

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Studies from our laboratory using hypomorphic apolipoprotein E (apoE) mice, also referred to as HypoE mice that carry the inducible Mx1-Cre transgene (*Apoe*$^{h/h}$Mx1-Cre mice), have shown previously that apoE can promote the regression of atherosclerosis beyond reducing plasma cholesterol levels.[15] The present study made use of HypoE mice to identify mechanisms involved in lesion size reduction and stabilization and the impact of hyperglycemia on these processes. We hypothesized that hyperglycemia impairs atherosclerosis regression by modulating the biological function of lesional macrophages. We found that hyperglycemia impairs lesion size reduction by hindering lipid removal and macrophage cholesterol transport, but that it does not significantly affect loss of macrophages from the lesion or remodeling of the vascular wall.

Materials and Methods

Materials

All reagents were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Study Design and Generation of Hyperglycemic Mice

The generation of hypomorphic *Apoe*$^{h/h}$ Mx1-*Cre* transgenic mice expressing wild-type mouse apoE, also referred to as HypoE mice, has been previously described.[16,17] Briefly, the Mx1-*cre* transgene allows the removal of the neocassette after the induction of Cre recombinase with polyinosinic-polycytidylic ribonucleic acid (pIpC) and repair of the hypomorphic *Apoe* allele in all tissues.

Male mice were weaned at 21 days, housed in a 12-hour cycle barrier facility, and fed a chow diet containing 4.2% fat (Harlan Teklad, Madison, WI). At 8 weeks of age, all mice were fed a high-fat diet (HFD) containing 1.25% cholesterol, 3.5% coconut oil, 7.5% cocoa butter, and 0.5% sodium cholic acid (Research Diets Inc., New Brunswick, NJ) for 18 weeks (baseline group). After 17 weeks of HFD, regressed groups of mice received an i.p. injection of either 70 mg/kg streptozotocin (STZ) or saline for 5 consecutive days. To avoid weight loss commonly associated with STZ-induced diabetes,[18] mice received 0.1 U of recombinant human insulin i.p. (Eli Lilly, Indianapolis, IN) every other day. After 18 weeks of HFD, regressed groups of mice were fed a low-fat diet (LFD) and given a 250 μg i.p. injection of pIpC. All procedures were in accordance with the NIH guidelines and the San Francisco VA Medical Center committee for animal care and welfare.

Blood Glucose, Body Weight, and Glycated Hemoglobin

Blood was collected via the tail vein, and fasting blood glucose (FBG) levels were measured with an AlphaTrack blood glucose monitoring system (Abbott Laboratories, Green Oaks, IL). FBG and body weights were measured every other day. HbA_{1c} levels were measured with an A1CNow+ Multitest A1C system (Cardinal Health, Pasadena, CA) at sacrifice.

Tissue Collection

Overnight fasted mice were sacrificed after 18 weeks of HFD (baseline group) or after an extra 4 weeks of LFD (regressed groups). Mice were anesthetized with isoflurane inhalation or tribromoethanol (Avertin) injection, and bled by heart puncture. Mice were perfused via heart puncture with ice-cold PBS containing ProtectRNA RNase Inhibitor (1.5 mL/minute for 10 minutes). The aortic root was embedded in Tissue-Tek optimum cutting temperature cryosectioning compound (Sakura Finetek, Tokyo, Japan) and flash frozen in liquid nitrogen.

Plasma Lipid and Lipoprotein Fractionation

Plasma lipoproteins were fractionated by fast protein liquid chromatography (FPLC) on a Superose 6 GL 10/30 column (pooled plasma from four mice per group; GE Healthcare, NJ). Plasma and FPLC fraction cholesterol and triglycerides were measured by colorimetric assays, according to the manufacturer's instructions (Cholesterol E, L-Type TG M; Wako, Chemicals, Richmond, VA) with a VersaMax microplate reader (Molecular Devices Corporation, Sunnyvale, CA).

Western Blot Analysis of Apolipoproteins

Plasma and FPLC fractions were subjected to SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA). Western blots were incubated with primary antibodies, goat anti-mouse apoA1 (GenWay Biotech Inc., San Diego, CA), rabbit anti-mouse apoE,[19] and rabbit anti-mouse apolipoprotein B (apoB),[16] followed by detection with IRDye 680 LT donkey anti-rabbit antibody or IRDye 800 CW donkey anti-goat antibody (LI-COR Biosciences, Lincoln, NE). Membranes were scanned and integrated intensity was quantified on an Odyssey infrared imaging system (LI-COR Biosciences).

Histological Quantification of Atherosclerosis

Tissue blocks were cut into sections (10 μm thick). Three cross sections (50 μm apart) of the aortic root were stained with oil red O and counterstained with hematoxylin. Adjacent sections were stained with Sirius red and counterstained with Fast green. Slides were mounted on a Zeiss AxioObserver Z1 microscope (Carl Zeiss Microimaging Inc., Thornwood, NY), and images were captured with a Retiga-SRV charge-coupled device camera equipped with an RGB color filter (Qimaging, Surrey, BC, Canada). Images were quantified with Metamorph software (Molecular Devices Corporation). Collagen types (I and III) were visualized with a circular polarizer.

Immunofluorescence Characterization of Aortic Root Lesions

Formalin-fixed and glycine-quenched cross sections of the aortic root were permeabilized with 0.5% saponin in PBS. Non-specific labeling was blocked with 10% donkey serum PBS for 2 hours at room temperature. Aortic root sections were incubated overnight at 4°C with a combination of antibodies, including rat anti−Mac-2 (Cedarlane Labs Ltd, Burlington, ON, CA), rabbit anti-apoE, rabbit anti-apoB, goat anti-apoA1, Cy3 mouse anti-smooth muscle α-actin, and goat anti-human fibronectin (Santa Cruz Biotechnology Inc., Santa Cruz, CA) in antibody buffer solution (solution of sodium citrate) with 2% donkey serum, 0.1% bovine serum albumin, and 0.1% saponin). After three washes with 0.1% saponin solution of sodium citrate, sections were incubated with either Alexa488-conjugated donkey anti-rat or anti-goat or Alexa594-conjugated anti-rabbit secondary antibodies (Life Technologies, Grand Island, NY) for 2 hours at room temperature. After three more washes, nuclei were stained with Hoechst 33342 (Life Technologies) and slides were mounted in SlowFade Gold (Life Technologies). Images of up to triple labels were acquired with a 5× objective and processed as previously described.

Blood Leukocyte Analysis

Blood samples were collected by retro-orbital puncture in EDTA and kept at room temperature. Aliquots of 50 μL of blood were incubated for 10 minutes with Fc receptor block (1:500). Leukocytes were labeled with the following antibodies for 20 minutes: PERCP/CY5.5-CD45, PE-Ly6G, and PE-CD3e (BioLegend, Inc., San Diego, CA); and FITC-CD11b, FITC-CD45R/B220, APC-CD62L, FITC-Ly6C, and PE-CD115 (BD Pharmingen, San Diego, CA). Red cells were then lysed, and samples were fixed and diluted with BD Red Cell Lysing Solution (BD Pharmingen). Samples were analyzed with a C6 Flow Cytometer (Accuri Cytometers Inc., Ann Arbor, MI) and FlowJo software version 7.6 (Tree Star Inc., Ashland, OR).

LCM of Lesional Macrophages

All laser-capture microdissection (LCM) procedures were performed under RNase-free conditions. The aortic root was divided into sections (20 μm thick) and mounted on Membrane Slides 1.0 PEN (Carl Zeiss Microimaging Inc.). Every fifth section was mounted on SuperFrost Plus slides (Fisher Scientific, Waltham, MA) and stained for MOMA-2 (guide slides; Cedarlane Labs Ltd). Sections for LCM were fixed in 70% ethanol, rinsed in water, counterstained with hematoxylin QS, rinsed in water, dehydrated in graded ethanol, and air dried. Macrophage areas were identified from guide slides and quickly microdissected (<30 minutes) with the P.A.L.M. system (Carl Zeiss Microimaging Inc.).

RNA Extraction and Gene Expression

Total RNA was extracted from microdissected macrophages using RNeasy Micro kits and on-column DNase I treatments, according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA concentration was determined by the Quant-iT RiboGreen RNA Kit (Life Technologies), and RNA quality was verified with the 2100 Bioanalyzer and RNA 6000 Pico Kit (Agilent, Santa Clara, CA). RNA integrity number averaged 7.93 and ranged between 7.0 and 8.8.

Microdissected macrophages from two to three mice were pooled and 10 ng of total RNA per sample was reverse transcribed and amplified using the RT^2 PreAMP cDNA Synthesis Kit (SABiosciences, Qiagen), according to the manufacturer's instructions. Quantitative RT-PCR analysis was performed using the Mouse Wound Healing RT^2 Profiler PCR array (catalog no. PAMM-121Z; SABiosciences, Qiagen), according to the manufacturer's instructions. A total of 84 key genes central to the wound-healing response were analyzed. Data analysis was performed using the manufacturer's integrated web-based software package for the PCR Array System using $2^{-\Delta\Delta C_T}$ based fold-change calculations (SABiosciences). Expression levels of candidate genes were also assessed using retro-transcribed nonamplified RNA, using an ABI Prism 7900 (Applied Biosystems, Foster City, CA) and in-house−designed primer pairs (SYBR Green reactions) or Assay-On-Demand (Applied Biosystems). Each target gene expression was normalized to the average housekeeping genes (cyclophilin, Tata box protein, β-2-microglobulin, or heat shock protein 90-β), and levels were calculated according to the $2^{-\Delta C_T}$ or $2^{-\Delta\Delta C_T}$ methods.

Statistical Analysis

Data were analyzed with GraphPad Prism software version 5 (GraphPad Software Inc., La Jolla, CA) using one- and two-way analyses of variance, followed by Bonferroni posttests or two-tailed Student's *t*-tests. A difference with a $P < 0.05$ was considered significant.

Results

Metabolic Parameters

The study design is shown in Figure 1A. Diabetes was confirmed by a threefold increase in FBG levels (578.7 ± 32.9 mg/dL versus 195.6 ± 11.5 mg/dL; $P < 0.001$) (Figure 1B) and a nearly twofold increase in the percentage of glycated HbA_{1c} (8.0% ± 0.2% versus 4.7% ± 0.1%; $P < 0.0001$) (Figure 1C) compared with saline-treated groups. The STZ-treated mice maintained a slight, but significantly, lower body weight than the saline-treated mice (26.6 ± 0.4 g versus 33.1 ± 0.6 g; $P < 0.001$) but never lost >15% of their baseline body weights (results not shown). No significant differences in FBG ($P = 0.37$) and plasma lipid (cholesterol, $P = 0.47$; triglycerides, $P = 0.60$) levels were

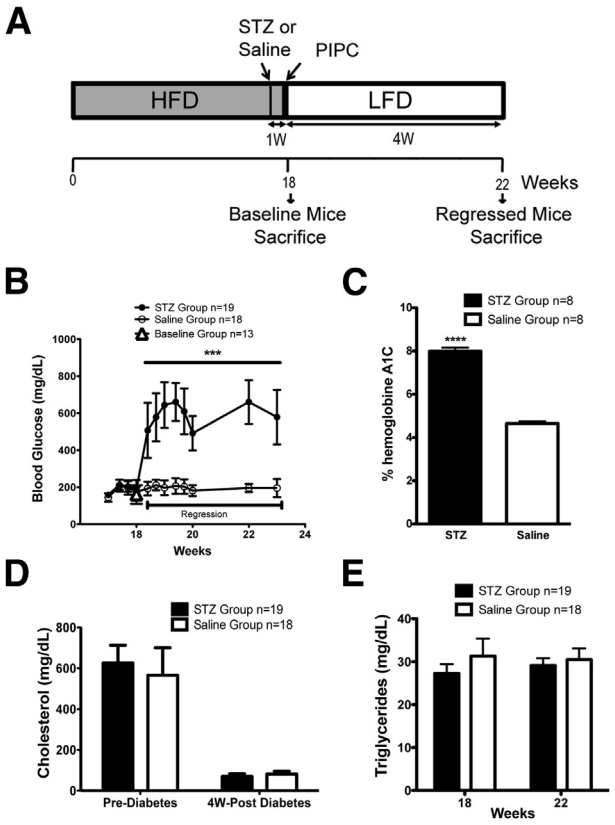

Figure 1 Experimental design and metabolic parameters. **A**: Hypomorphic *apoE* mice carrying the *Mx1-cre* transgene (*Apoe*$^{h/h}$ MX1-*Cre*) were fed a high-fat diet (HFD) for 18 weeks. All mice received either a series of five consecutive i.p. injections of saline (baseline and saline groups) or streptozotocin (STZ group) during the 17th week of HFD. A group of mice was sacrificed after 18 weeks of HFD and used as the baseline group. The two other groups of mice were given an i.p. injection of pIpC and switched to a low-fat diet (LFD) after 18 weeks of HFD. These two groups of mice were sacrificed 4 weeks after the pIpC injection and consumption of a chow diet. Blood glucose levels over time (**B**) and percentage of HbA_{1c} at sacrifice (**C**) for both the saline and STZ groups. Plasma cholesterol (**D**) and triglyceride (**E**) levels before (average of weeks 17 and 18) and 4 weeks after the induction of diabetes. Two-way analysis of variance, followed by Bonferroni posttests: ***$P < 0.001$, ****$P < 0.0001$.

found between the two groups at baseline before STZ treatment. The change to LFD and repair of the hypomorphic *Apoe* gene decreased plasma cholesterol levels by sevenfold (555.5 ± 30.3 to 81.1 ± 3.3 mg/dL in the saline group; $P < 0.0001$) within 1 week of pIpC. Reductions in plasma cholesterol occurred at a similar rate among the STZ group of mice treated with pIpC. No differences in plasma cholesterol or triglyceride levels were noted between the saline- and STZ-treated mice for the remaining 4 weeks (analysis of variance, $P > 0.05$) (Figure 1, D and E).

The change to LFD and repair of the hypomorphic *Apoe* gene significantly modified, at the same rate, the lipid profiles for both groups of mice. At baseline, most of the plasma cholesterol was distributed to very-low-density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and LDL fractions. After 4 weeks of lipid lowering, VLDL- and IDL-LDL-cholesterol levels were dramatically decreased, whereas high-density lipoprotein (HDL) cholesterol levels were maintained in both saline- and STZ-treated groups (Figure 2A). Apolipoprotein B (apoB100 and apoB48) was on the VLDL, IDL, and LDL fractions at both baseline and 4-week regression time points. ApoA1, found in VLDL-LDL and HDL fractions at baseline, was located almost exclusively in the HDL fractions at the 4-week regression time point. ApoE was located mostly in VLDL-LDL fractions at baseline and 4-week regression time points but was also seen in early HDL fractions after 4 weeks of regression (Figure 2B). No differences in the apolipoprotein distribution were found between the saline- and STZ-treated groups. The relative amounts of plasma apoB100, apoB48, apoA1, and apoE were reduced by 34%, 82%, 19%, and 60%, respectively, after 4 weeks of regression in both groups of mice (Figure 2C). This produced an increase of 86% in the plasma apoA1/apoB ratio.

Hyperglycemia Impairs Atherosclerosis Regression

Four weeks of sustained lipid lowering induced a significant 36% reduction in lesion size in the aortic roots of saline-treated mice ($P = 0.03$). In contrast, we observed only a 14% reduction in STZ-treated mouse lesion size ($P = 0.30$) (Figure 3A). The regressed lesions were characterized by a decrease in neutral lipid content significantly greater in saline- than STZ-treated mice (38% versus 26%; $P = 0.02$) (Figure 3, B and C). We first wondered if the impaired lipid loss seen in the lesions of hyperglycemic mice was associated with an increase in proteoglycan-mediated retention of atherogenic apoB-containing lipoproteins. Thus, we assessed the apolipoprotein content of the lesions (Figure 3, D–F). Although the relative lesion content of apoA1 and apoE remained the same after 4 weeks of regression, the apoB content decreased significantly by 63% ($P = 0.002$) and 55% ($P = 0.0006$) in saline- and STZ-treated mice, respectively. These changes contributed to increase the apoA1/apoB ratio by nearly twofold in the regressed lesions of both groups of mice. Unexpectedly, hyperglycemia did not affect significantly the relative apoB content or the apoA1/apoB ratio of the regressed aortic lesions ($P = 0.34$) (Figure 3F).

We next tested the possibility that the impaired lipid loss from the lesion was due to a macrophage-mediated decrease in lipid removal. Quantification of macrophage marker, Mac-2, revealed a significant decrease in macrophage content in saline- and STZ-treated lesions, 41% ($P < 0.0001$) and 32% ($P < 0.001$), respectively, compared with baseline (Figure 3, G and H). However, the difference between saline- and STZ-treated mice did not reach statistical significance ($P = 0.24$). Next, we tested whether hyperglycemia altered lesional macrophage gene expression that could have affected their function in remodeling atheroma. To this end, we used LCM to isolate macrophages from aortic roots and assessed the expression levels of genes associated with cholesterol transport. The expression level of all genes tested decreased after regression and by at least twofold for ATP-binding cassette A1 (*ABCA1*) and ATP-binding

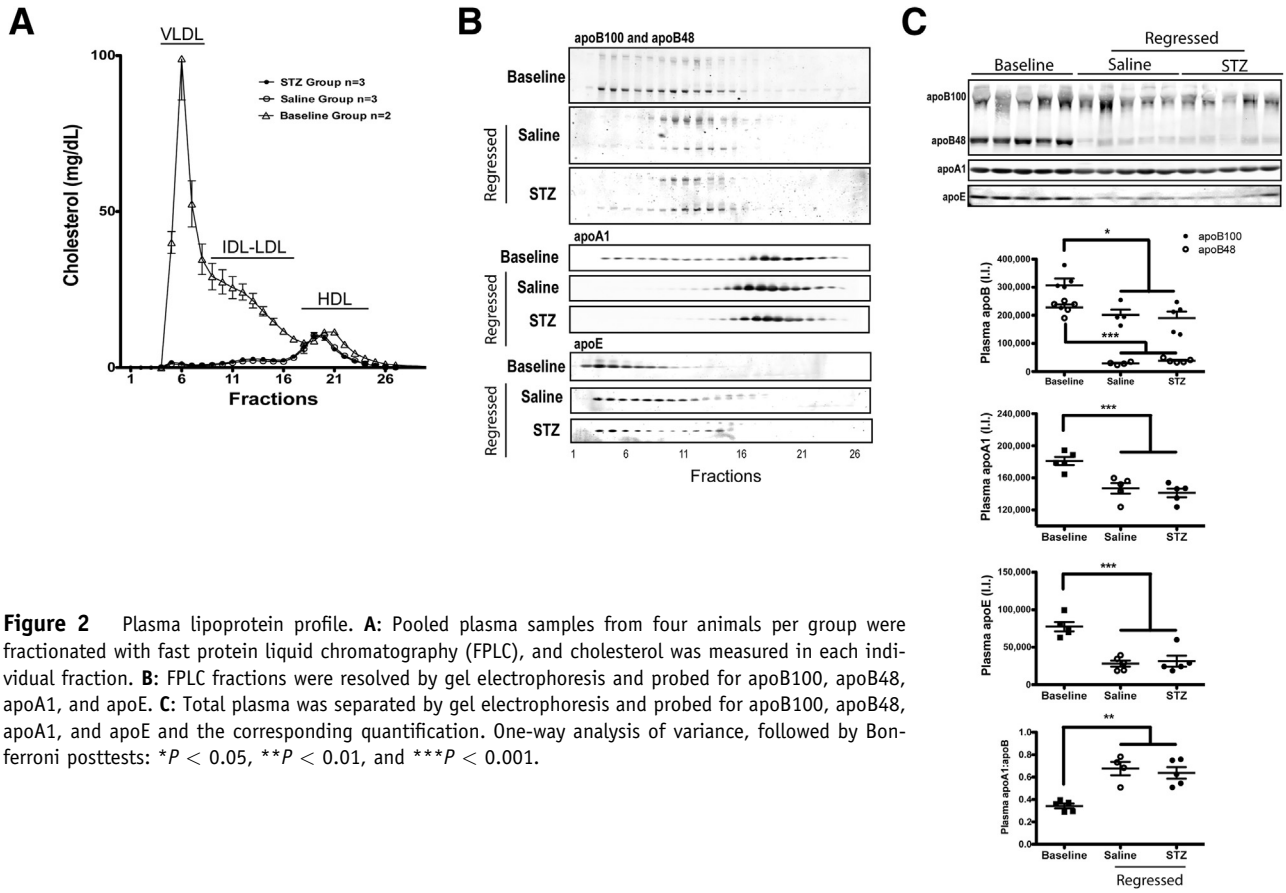

Figure 2 Plasma lipoprotein profile. **A:** Pooled plasma samples from four animals per group were fractionated with fast protein liquid chromatography (FPLC), and cholesterol was measured in each individual fraction. **B:** FPLC fractions were resolved by gel electrophoresis and probed for apoB100, apoB48, apoA1, and apoE. **C:** Total plasma was separated by gel electrophoresis and probed for apoB100, apoB48, apoA1, and apoE and the corresponding quantification. One-way analysis of variance, followed by Bonferroni posttests: $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

cassette G1 (*ABCG1*). We also found that hyperglycemia resulted in the down-regulation of ABCA1, ABCG1, scavenger receptor class B1 (SRB1), and scavenger receptor class B family member (CD36; CD36 and SRB1 increased by 1.75- and 1.2-fold, respectively, compared with baseline) gene expression levels (Table 1).

Effects of Hyperglycemia on Inflammatory Circulating and Lesional Cells

To test whether hyperglycemia had an effect on the number and subtype of circulating inflammatory cells, we performed whole blood flow cytometry analysis. In the saline-treated group, total leukocyte counts remained constant between baseline and 4-week regression time points. However, total leukocyte counts in STZ-treated mice increased significantly by 25% compared with saline-treated mice ($P = 0.04$) (Figure 4A). This increase in total leukocytes was partly the result of a 40% greater increase in B-cell count in STZ-treated mice compared with both baseline and saline-treated mice (Figure 4B). STZ-treated mice also had a lower granulocyte cell count compared with saline-treated mice. We next tested the possibility that hyperglycemia and lipid lowering altered the activation of circulating monocytes but found no significant difference in the total count of Ly6C^{high} and Ly6C^{low} monocytes ($P = 0.65$ and $P = 0.34$) or in the expression level of CD62L on Ly6C^{high} monocytes ($P = 0.46$) (Figure 4, C–E).

In addition, we assessed expression levels of genes associated with M1/inflammatory and M2/anti-inflammatory phenotypes in microdissected macrophages and found no significant differences between baseline and the two regressed groups (Table 2).

Lipid Lowering and Hyperglycemia Modulate the Expression of Genes Associated with Wound Healing in Lesional Macrophages

Atherosclerosis regression involves a remodeling of the vascular wall resembling that of the wound-healing response, a pathway compromised in diabetic patients.[20] To test whether this pathway is involved in atherosclerosis regression and impaired by hyperglycemia, we analyzed the expression of a panel of genes in microdissected macrophages, using pathway-focused PCR arrays. The array was composed of 84 key genes central to the wound-healing response. Subsets of these genes modulate processes, including extracellular matrix remodeling, cellular adhesion, cellular growth, and signal transduction. Nearly a third of the genes analyzed (with a cycling threshold >30) were significantly up-regulated by at least twofold after 4 weeks of regression in lesional macrophages of saline-treated mice

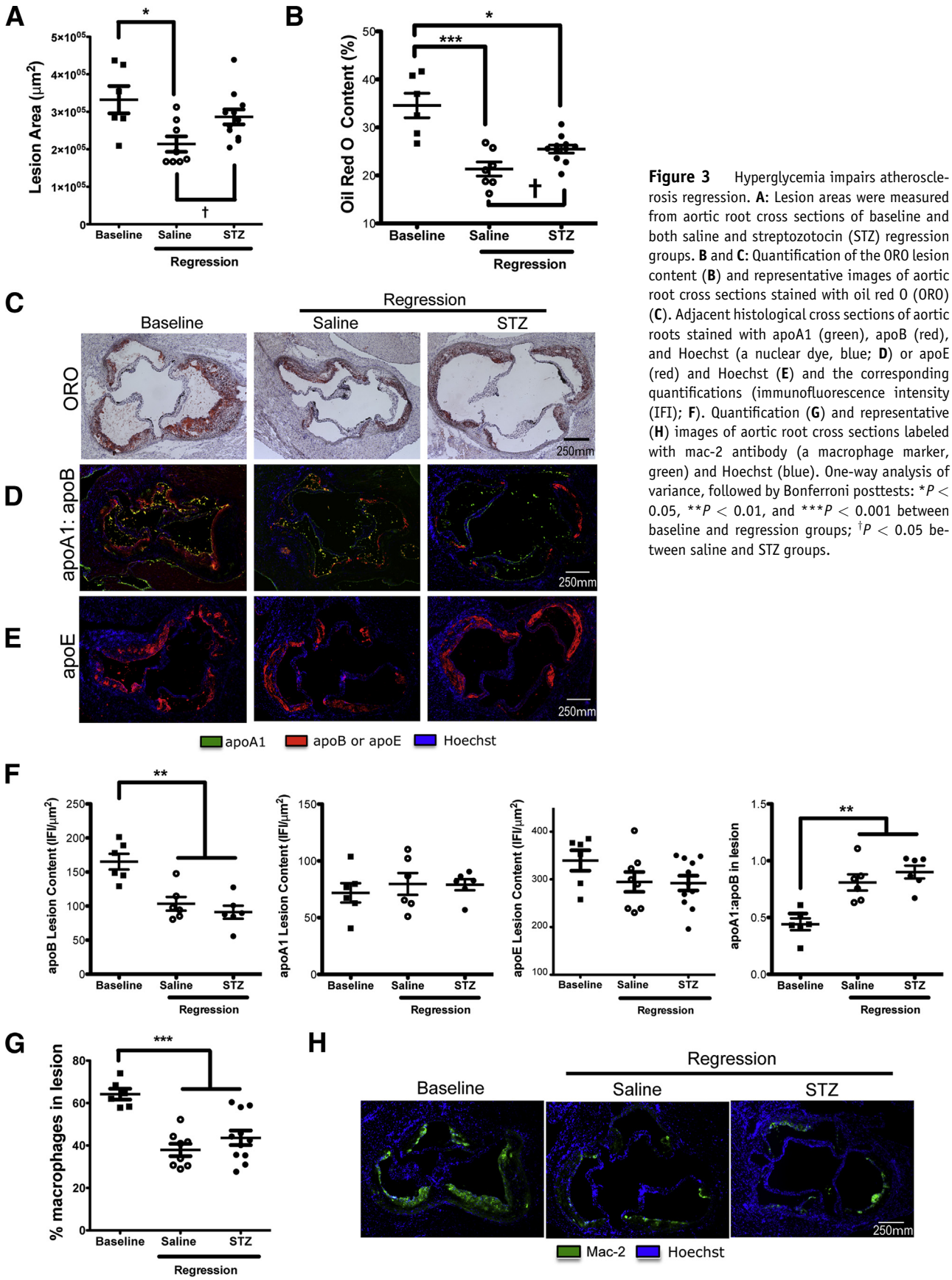

Figure 3 Hyperglycemia impairs atherosclerosis regression. **A:** Lesion areas were measured from aortic root cross sections of baseline and both saline and streptozotocin (STZ) regression groups. **B** and **C:** Quantification of the ORO lesion content (**B**) and representative images of aortic root cross sections stained with oil red O (ORO) (**C**). Adjacent histological cross sections of aortic roots stained with apoA1 (green), apoB (red), and Hoechst (a nuclear dye, blue; **D**) or apoE (red) and Hoechst (**E**) and the corresponding quantifications (immunofluorescence intensity (IFI); **F**). Quantification (**G**) and representative (**H**) images of aortic root cross sections labeled with mac-2 antibody (a macrophage marker, green) and Hoechst (blue). One-way analysis of variance, followed by Bonferroni posttests: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ between baseline and regression groups; $^{\dagger}P < 0.05$ between saline and STZ groups.

Table 1 Gene Expression Analysis of Lesional Macrophages Focused on the Cholesterol Influx/Efflux Pathway

Variable	Baseline	*n*	Regressed/saline	*n*	Regressed/STZ	*n*
LXRα	0.52 ± 0.08	4	0.39 ± 0.04	4	0.39 ± 0.08	4
ABCA1	8.99 ± 2.16	4	4.33 ± 1.12	4	9.59 ± 1.74*†	4
ABCG1	2.88 ± 0.98	4	1.17 ± 0.24	4	2.45 ± 0.11‡	4
SRB1	0.20 ± 0.07	4	0.16 ± 0.08	4	0.24 ± 0.03†§	4
CD36	5.33 ± 0.85	4	4.23 ± 1.77	4	9.33 ± 1.62*¶	4

Data are presented as relative mRNA level normalized to the average of two housekeeping genes: cyclophilin and Tata box protein (means ± SEM and sample size).

*$P < 0.01$ between saline- and STZ-treated regressed groups (data analyzed with two-way analysis of variance, followed by Bonferroni posttests).

†$P < 0.05$ (data analyzed with *t*-test).

‡$P < 0.01$ between saline- and STZ-treated mice (data analyzed with *t*-test).

§$P < 0.05$ between baseline and STZ-treated group (data analyzed with *t*-test).

¶$P < 0.05$ between baseline and regressed groups (data analyzed with two-way analysis of variance, followed by Bonferroni posttests).

LXR, liver X receptor.

(Table 3). These included five types of collagens, the remodeling enzyme matrix metalloproteinase 2 (MMP-2), cellular adhesion integrin-α3, connective tissue, fibroblast growth factors, and prostaglandin-endoperoxide synthase 2 (cyclooxygenase-2). In addition, and of particular interest, was the significant 2.56-fold down-regulation of the remodeling enzyme, cathepsin L. By comparison, the array data revealed that hyperglycemia blunted the up-regulation of most collagen genes (types IV/V) and *Mmp2* and the down-regulation of cathepsin L, while preserving the modulation of other genes. Hyperglycemia also modulated the expression of genes in lesional macrophages that remained unchanged in the euglycemic group of regressed mice. These included a more than twofold up-regulation of cathepsin K, integrin-β3, and transgelin. These results indicate that lipid lowering modulates the expression level of genes associated with the wound-healing response in lesional macrophages and that hyperglycemia alters this process.

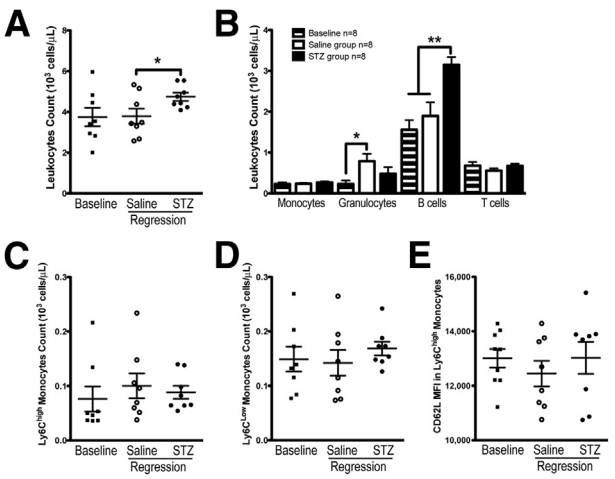

Figure 4 Flow cytometry analysis of circulating leukocytes in saline control and streptozotocin (STZ)-treated mice at baseline and after 4 weeks of regression. Leukocyte ($CD45^+$ cell) counts (**A**) and percentage of leukocytes identified as monocytes ($CD115^+$ cells), granulocytes ($CD115^-$, $Ly6G^+$ cells), B cells ($B220R^+$ cells), and T cells ($CD3^+$ cells) (**B**). Monocyte subtype $Ly6C^{high}$ (**C**) and $Ly6C^{low}$ (**D**) counts and the expression level of CD62L (L-selectin) on $Ly6C^{high}$ monocytes (**E**). Mean fluorescence intensity (MFI): *$P < 0.05$, **$P < 0.01$ by one-way analysis of variance (**A**) and two-way analysis of variance (**B**), followed by Bonferroni posttests.

Effect of Hyperglycemia on Regressed Lesion Composition

Results of the array experiment suggested an impact of hyperglycemia on the remodeling of the regressed lesion. Consequently, we examined the content of collagen, smooth muscle cell (SMC), and fibronectin in lesions of baseline and regressed mice, as an index of lesion stabilization. Unexpectedly, we observed a similar 64% increase in total collagen content ($P = 0.26$, mostly type I fibers) (Figure 5C) in lesions of saline- and STZ-treated mice (Figure 5, A–C). SMC content was scarce and diffuse in baseline lesions (1.2% ± 0.3%) but more abundant and forming thin fibrous caps in regressed lesions of both saline- and STZ-treated mice (4.2% ± 1.0% versus 5.0% ± 1.4%; $P = 0.66$) (Figure 5, D and E). Fibronectin content in lesions decreased by 47% and 32% in saline- and STZ-treated mice, respectively ($P = 0.54$) (Figure 5, E and F). Taken together, these results suggest that hyperglycemia did not significantly affect the remodeling and stabilization of atherosclerotic lesions.

Discussion

We previously reported the regression of advanced diet-induced atherosclerotic lesions in HypoE ($Apoe^{h/h}$Mx1-Cre) mice induced to express normal apoE levels and switched to a chow diet.[15] In this study, we aimed to identify mechanisms involved in the regression of atherosclerosis and to determine the impact of hyperglycemia on this process. To this end, we induced hyperglycemia in HypoE mice with preexisting atherosclerotic lesions, reversed hyperlipidemia, and assessed gene expression in lesional macrophages after 4 weeks of lipid lowering. The main finding of this study is that hyperglycemia impairs lesion size reduction. Our results also show that hyperglycemia results in more lipid retention and/or accumulation within atheroma after sustained plasma

Table 2 Gene Expression Analysis of Lesional Macrophage Phenotypes

Variable	Baseline	*n*	Regressed/saline	*n*	Regressed/STZ	*n*
Migration						
CCR7	0.051 ± 0.002	3	0.031 ± 0.015	4	0.041 ± 0.014	4
M1/M2 phenotype						
iNOS	0.038 ± 0.013	4	0.063 ± 0.027	4	0.033 ± 0.005	4
ARG1	0.002 ± 0.001	3	0.010 ± 0.008	3	0.008 ± 0.006	3
CD86	0.090 ± 0.013	4	0.073 ± 0.019	4	0.105 ± 0.012	4
HB-EGF	0.215 ± 0.015	4	0.255 ± 0.058	4	0.274 ± 0.034	4
LIGHT	0.016 ± 1.352	4	0.008 ± 0.003	4	0.013 ± 0.002	4

Data are presented as relative mRNA level normalized to the housekeeping gene *Hsp90ab1* (means ± SEM and sample size). Data were analyzed with two-way analysis of variance and *t*-test.

ARG, arginase; CCR, chemokine receptor; HB-EGF, heparin-binding epidermal growth factor–like growth factor; iNOS, inducible nitric oxide synthase; LIGHT, tumor necrosis factor superfamily-14.

lipid lowering. Accordingly, a gene expression analysis revealed that hyperglycemia altered the expression of genes associated with cholesterol homeostasis in lesional macrophages that could have contributed to more abundant cellular lipid. Our data also suggest the involvement of lesional macrophages in the healing response of the vascular wall and that hyperglycemia only has a mild impact on this response and overall remodeling.

Although there is a clear association between diabetes and increased risk for cardiovascular diseases, an independent role of hyperglycemia in the development of atherosclerosis has remained controversial. This is mainly the result of hyperglycemia-mediated hyperlipidemia that, in turn, exacerbates atherosclerosis.[14]

In mouse models, STZ treatment has been identified to decrease the expression of sulfation enzymes that normally add negative charges to proteoglycans on liver hepatocytes.[21] This reduces the trapping of remnant lipoproteins in the liver of hyperglycemic mice deficient in *Apoe* or the LDL receptor–fed HFD, resulting in added hyperlipidemia.[21,22] In this study, both STZ- and saline-treated mice displayed similar plasma lipid levels and lipoprotein profiles during the 4 weeks of sustained plasma lipid lowering owing to the repair of the hypomorphic *Apoe* gene and to the switch to LFD.

Previous studies have reported that hyperglycemia impairs remodeling and stabilization of atherosclerotic lesions.[23,24] To our knowledge, our study is the first to report that hyperglycemia impairs lesion size reduction. Johansson et al[24] described the effect of hyperglycemia in $LDLR^{-/-}$ mice fed an HFD for 16 weeks, followed by subsequent LFD for 14 weeks. Diabetes was induced after 2 weeks of LFD. In this model, hyperglycemia was induced on aggressive plasma lipid lowering achieved with a helper-dependent adenovirus to stably overexpress the VLDL receptor in the liver. The overexpression of the VLDL receptor allowed complete normalization of plasma cholesterol and triglyceride levels. The authors found that, in advanced atherosclerotic lesions, hyperglycemia increased plaque disruption through a mechanism that was dependent on elevated levels of triglyceride-rich lipoproteins.[24]

A more recent study, reported by Parathath et al,[23] investigated the effect of hyperglycemia on atherosclerosis regression in the Reversa mouse model. In this mouse model, 16 weeks of HFD produced advanced atherosclerotic lesions. Hyperlipidemia was then reversed by switching to an LFD and by inactivation of the gene encoding the microsomal triglyceride transfer protein. In this mouse model, lipid lowering induced remodeling of atherosclerotic lesions without changing lesion size. The authors also reported that hyperglycemia impaired the remodeling of atherosclerotic lesions independently of triglyceride levels.[23] In both Johansson's and Parathath's models, lesions developed in the presence of high plasma LDL-cholesterol and triglyceride levels. In contrast, in our model, atherosclerosis developed in the presence of high plasma VLDL-cholesterol and low plasma triglyceride levels. Consequently, although initial atherosclerotic lesion sizes were similar between studies, the lesions in our study had a greater content in neutral lipid, macrophages, and collagen. Thus, the composition of advanced lesions may affect the process of regression induced by plasma lipid lowering.[25] The lipid profile is yet another important factor that may modulate lesion regression.[26–28] In contrast to Parathath et al,[23] our mice regressed in the presence of an increased ratio of plasma HDL/LDL and an overall higher plasma HDL-cholesterol level. In our model, the regressed lesions were characterized by a decrease in neutral lipid, apoB-containing lipoproteins, and macrophage contents. However, hyperglycemia only impaired the loss in neutral lipid content.

The response to retention hypothesis implicates the interactions of apoB lipoproteins with proteoglycans of the subendothelial extracellular matrix.[29] Diabetes has been linked to increased synthesis of proteoglycans in the vascular wall.[30,31] In addition, the glycation of lipoproteins has been shown to increase their binding affinity for proteoglycans.[32] Based on the literature, we expected hyperglycemia to increase lipoprotein retention in the lesions. Although we observed a higher fibronectin content in the regressed lesions of hyperglycemic mice, this increase did not correlate with greater lesional apoB presence. Thus, the impaired loss in lipid observed in regression is unlikely as the result of an increased retention of atherogenic

Table 3 Gene Expression Profile of Lesional Macrophages Reveals the Involvement of Genes that Participate in Wound Healing during Atherosclerosis Regression

Gene	Fold up- or down-regulation (baseline vs saline regressed)	*P* value	Fold up- or down-regulation (baseline vs STZ regressed)	*P* value	Gene description
Extracellular Matrix					
Col14a1	1.76	0.18	−1.06	0.77	Collagen, type XIV, α1
Col1a1	1.35	0.52	1.36	0.51	Collagen, type I, α1
Col1a2	2.69	0.003*	1.36	0.37	Collagen, type I, α2
Col3a1	1.41	0.06	−1.06	0.63	Collagen, type III, α1
Col4a1	2.00	0.001*	1.49	0.10	Collagen, type IV, α1
Col4a3	2.54	0.08	2.06	0.03*	Collagen, type IV, α3
Col5a1	2.46	0.04*	1.99	0.12	Collagen, type V, α1
Col5a2	2.31	0.04*	1.62	0.09	Collagen, type V, α2
Remodeling Enzymes					
Ctsk	1.80	0.05*	3.07	0.01*	Cathepsin K
Ctsl	−2.56	0.08	−1.40	0.28	Cathepsin L
Mmp2	2.81	0.01*	1.56	0.22	Matrix metallopeptidase 2
Plau	−1.13	0.44	−1.40	0.31	Plasminogen activator, urokinase
Plaur	−1.09	0.89	1.11	0.61	Plasminogen activator, urokinase receptor
Cellular Adhesion					
Itga3	3.31	0.09	2.82	0.01*	Integrin α3
Itga5	1.39	0.23	1.41	0.26	Integrin α5 (fibronectin receptor α)
Itga6	−3.24	0.01*	−2.42	0.08	Integrin α6
Itgav	−1.33	0.78	1.18	0.50	Integrin α5
Itgb1	−1.15	0.50	1.04	0.96	Integrin β1 (fibronectin receptor β)
Itgb3	−1.43	0.50	3.56	0.14	Integrin β3
Itgb5	1.30	0.28	1.34	0.20	Integrin β5
Cytoskeleton					
Rac1	−1.36	0.55	−1.18	0.72	RAS-related C3 botulinum substrate 1
Rhoa	1.16	0.19	1.08	0.56	RAS homolog gene family, member A
Tagln	1.55	0.15	2.42	0.11	Transgelin
Growth Factors					
Pdgfa	1.14	0.57	1.37	0.43	Platelet-derived growth factor, α
Tgfb1	−1.17	0.89	−1.05	0.85	Transforming growth factor, β1
Ctgf	2.16	0.01*	2.72	0.01*	Connective tissue growth factor
Fgf2	3.41	0.01*	2.89	0.12	Fibroblast growth factor 2
Hgf	1.76	0.27	−1.53	0.16	Hepatocyte growth factor
Igf1	−1.44	0.24	1.05	0.92	Insulin-like growth factor-1
Signal Transduction					
Mapk1	−1.02	0.69	−1.07	0.86	Mitogen-activated protein kinase 1
Mapk3	1.09	0.59	1.60	0.18	Mitogen-activated protein kinase 3
Pten	1.03	0.76	1.19	0.27	Phosphatase and tensin homolog
Il6st	1.87	0.06	1.36	0.27	IL-6 signal transducer
Ctnnb1	1.26	0.37	−1.31	0.58	Catenin (cadherin associated protein), β1
Ptgs2	3.02	0.01*	2.71	0.03*	Prostaglandin-endoperoxide synthase 2

*$P < 0.05$.

lipoproteins. However, we cannot exclude the possibility that a longer period of hyperglycemia may induce sufficient glycation of atherogenic plasma lipoproteins and increase their retention in the lesion.

Lipid loss from atherosclerotic lesions has been attributed to an increase in HDL-dependent reverse cholesterol transport.[10] Interestingly, nonenzymatic glycosylation of HDL has been shown to decrease its ability to promote cholesterol efflux[33] and, thus, could explain the impaired lipid loss observed in the lesion of hyperglycemic mice. In macrophages, several receptors mediate cholesterol transport. High glucose has been shown to increase the expression and activity of CD36[34] and SRB1,[35] leading to higher influx, while suppressing that of ABCG1[34] and ABCA1,[36] diminishing the efflux. We analyzed gene expression levels of these mediators of cholesterol transport in microdissected macrophages. A sustained 4-week period of lipid lowering led to lower expression levels of ABCA1 and ABCG1 by at least twofold in euglycemic mice. In contrast, in the presence of hyperglycemia, ABCA1 and ABCG1

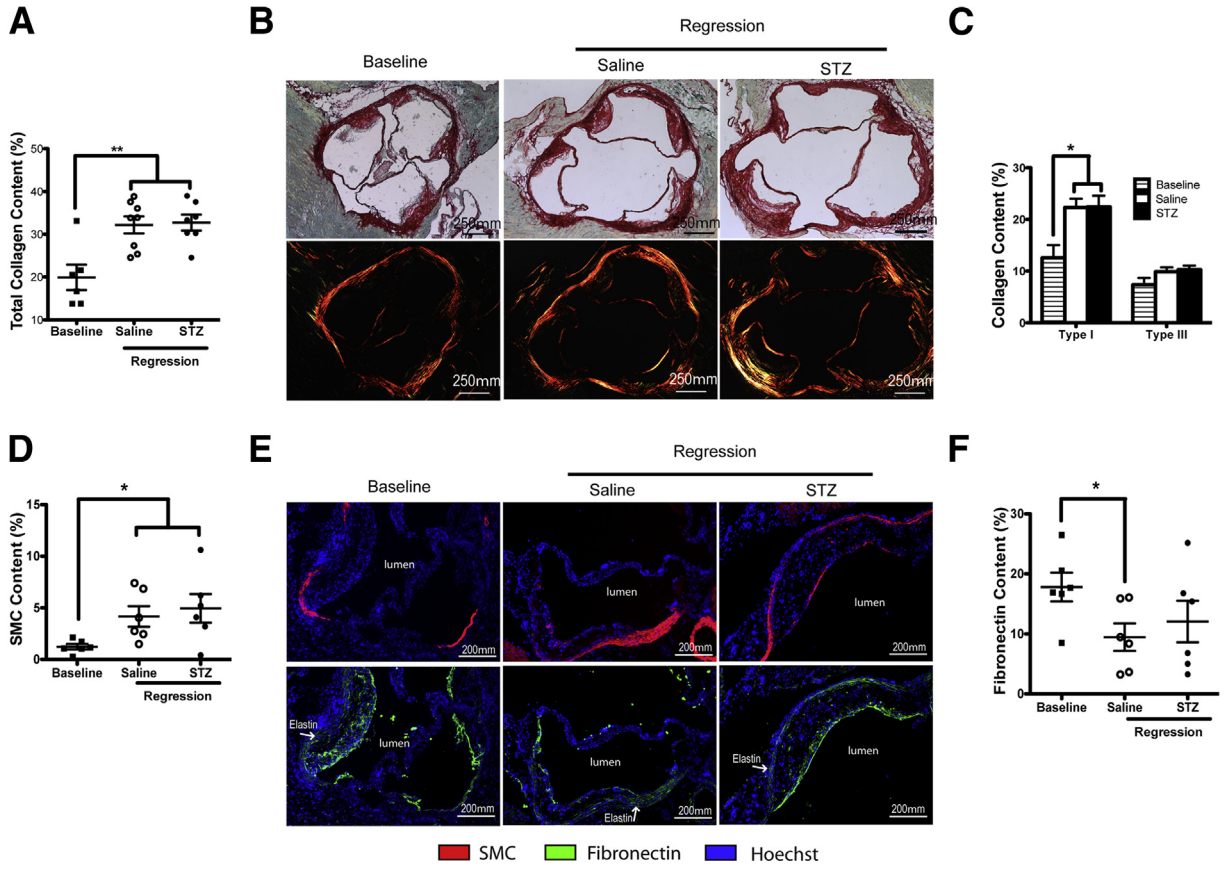

Figure 5 Hyperglycemia does not impair lesion remodeling during atherosclerosis regression. Quantification of total collagen (**A**) and representative images of cross sections of aortic roots stained with Sirius red (bright-field and polarized images) (**B**). **C**: Quantification of collagen type I (bright orange-yellow polarized fibers) and collagen type III (green polarized fibers) is shown. Quantification (**D**) of smooth muscle cell (SMC) content of the lesion and representatives images of aortic root cross sections labeled with anti—SMC-α-actin antibody (red) and Hoechst (blue; **E**, top). Quantification (**F**) of fibronectin content of the lesion and representatives images of aortic root cross sections labeled with anti-fibronectin antibody (green) and Hoechst (blue; **E**, bottom). One- and two-way analysis of variance, followed by Bonferroni posttests: $*P < 0.05$, $**P < 0.01$ between baseline and regression groups.

expression levels remained unchanged, whereas SRB1 and CD36 were significantly up-regulated. ABCA1 and ABCG1 expression levels are regulated by the interaction of oxysterols with the transcription factor, liver X receptor α.[37] Thus, lower levels of cellular oxysterols after 4 weeks of lipid lowering could explain the decrease in expression of ABCA1 and ABCG1 in lesional macrophages of euglycemic mice. Higher intracellular lipid levels in macrophages of hyperglycemic mice may have resulted in maintaining a higher expression of these transporters. It is possible that hyperglycemia-associated glycosylation of plasma proteins, including apoA1 in HDL, could have negatively affected reverse cholesterol transport in lesional macrophages, as previously suggested.[38] Taken together, these results suggest that hyperglycemia impaired lipid loss or enhanced lipid uptake in lesions by modulating macrophage reverse cholesterol transport or enhancing its import.

Hyperglycemia has been associated with increased systemic inflammation.[39] Four weeks of lipid lowering increased circulating granulocytes without affecting total leukocyte counts. Hyperglycemia hindered the increase in granulocytes but significantly increased total leukocyte and circulating B-cell counts. B cells are atheroprotective cells,[40] and their higher count in hyperglycemic mice unlikely explains the impairment of lesion regression. We cannot exclude the possibility that hyperglycemia or other consequential metabolic stress could have potentially modulated the inflammatory status of other cells[41] and increased systemic inflammation.[42] However, in our model of atherosclerosis regression, hyperglycemia did not modulate significantly the inflammatory state of circulating monocytes or lesional macrophages.

In addition to their involvement in lipid removal from the lesion, we demonstrate that lesional macrophages are active participants in the vascular healing response. The up-regulation of genes responsible for the synthesis of collagen and the down-regulation of enzymes promoting degradation of the extracellular matrix suggest that macrophages contribute to lesion stabilization and remodeling. The rupture-resistant nature of the regressed lesion was confirmed by increases in collagen, fibronectin, and fibrous cap formation. In contrast, the up-regulation of MMP-2 in our regression model is intriguing. MMP-2 is typically seen as a pro-atherogenic enzyme[43] associated with unstable lesions.[44] Indeed, MMP-2

is involved in the breakdown of collagen type IV. It is possible that its increased expression may have contributed to lesion size reduction. In this regard, regression studies that showed down-regulation of MMP-2 failed to observe a decrease in lesion size despite important lesion remodeling.[23] In this study, lesional macrophages of hyperglycemic mice expressed lower levels of MMP-2 and also had a significantly larger lesion size than euglycemic mice, providing compelling evidence for its participation in lesion shrinkage.

Although hyperglycemia attenuated changes in gene expression levels in lesional macrophages induced by lipid lowering, we found no such evidence at the protein level. Small increases in macrophage and SMC lesion content of hyperglycemic mice may have compensated for the lower gene expression levels. Alternatively, other cell types, such as endothelial cells and SMCs, could have contributed to the lesion remodeling. Limitations of our findings as they relate to atherosclerosis vascular disease in human diabetic individuals include the site of atherosclerosis, the duration of hyperglycemia, and the absence of obesity or even insulin treatment of our diabetic mouse model. It is possible that extended periods of hyperglycemia accompanied with enhanced systemic inflammation caused by obesity could render atheroma even more refractory to regression on therapeutic plasma lipid lowering. Alternatively, it is possible that regular insulin treatment to control hyperglycemia could alleviate lesional macrophage dysfunction and, thereby, enhance atherosclerosis regression in response to plasma lipid lowering. Last, it will be important to assess the impact of hyperglycemia on the regression of atherosclerosis in different vascular beds, including coronary and peripheral vessels, because findings derived from our study focused solely on atherosclerosis in the aortic root. These possibilities will be addressed in future studies of our model.

In conclusion, our study provides evidence that hyperglycemia impairs lesion regression and better control of hyperglycemia could enhance atherosclerosis regression. Based on the analysis of plaque composition and lesional macrophage gene expression, we propose that hyperglycemia impairs atherosclerosis regression by decreasing lipid removal from lesional macrophages through modulation of reverse cholesterol transport. Our results also suggest the participation of lesional macrophages in the vascular healing response, which is compromised by hyperglycemia. Thus, the development of therapies targeting biological functions of macrophages associated with both cholesterol transport and the healing response may enhance atherosclerosis regression both in the presence and absence of hyperglycemia.

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