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Exogenous insulin infusion can decrease atherosclerosis in diabetic rodents by improving lipids, inflammation, and endothelial function

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Abstract

Objective—To evaluate whether exogenously-induced hyperinsulinemia may increase the development of atherosclerosis.

Approach & Results—Hyperinsulinemia, induced by exogenous insulin implantation in high fat fed (60% fat HFD) ApoE$^{-/-}$ mice, exhibited insulin resistance, hyperglycemia and hyperinsulinemia. Atherosclerosis was measured by the accumulation of fat, macrophage, and extracellular matrix in the aorta. After 8 weeks on HFD, ApoE$^{-/-}$ mice were subcutaneously implanted with control (sham) or insulin pellet, and phlorizin, a SGLT1/2 inhibitor, for additional 8 weeks.

Intraperitoneal glucose tolerance test showed that plasma glucose levels were lower and insulin and IGF1 levels were 5.3 fold and 3.3 fold higher, respectively, in insulin implanted compared to sham-treated ApoE$^{-/-}$ mice. Plasma triglyceride, cholesterol and lipoprotein levels were decreased in mice with insulin implant, in parallel with increased lipoprotein lipase activities. Atherosclerotic plaque by *en face* and complexity staining showed significant reductions of fat deposits and expressions of VCAM-1, TNFα, IL6 and macrophages in arterial wall while exhibiting increased activation of pAKT and eNOS ($p<0.05$) comparing insulin implanted vs. sham HFD ApoE$^{-/-}$ mice. No differences were observed in atherosclerotic plaques between phlorizin treated and sham HFD ApoE$^{-/-}$ mice, except phlorizin significantly lowered plasma glucose and glycated hemoglobin levels while increased glucosuria. Endothelial function was improved only by insulin treatment through eNOS/NO activations and reduced pro-inflammatory (M1) and increased anti-inflammatory (M2) macrophages, which were inhibited by eNOS inhibitor.

Conclusions—Exogenous insulin decreased atherosclerosis by lowering inflammatory cytokines, macrophages, and plasma lipids in HFD induced hyperlipidemia, insulin resistant and mildly diabetic ApoE$^{-/-}$ mice.

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Disclosures

None

Keywords

Hyperglycemia; atherosclerosis; insulin resistance; diabetes mellitus

Subject Code

Vascular Disease

Introduction

Hyperinsulinemia is associated with increased risk for cardiovascular diseases (CVDs) [1, 2] which are the major causes of morbidity and mortality among people with insulin-resistance and diabetes. Hyperinsulinemia is present in systemic insulin resistance or exogenous insulin treatments[3]. The associations of systemic insulin resistance and endogenous hyperinsulinemia with elevated risk of CVDs have been consistently shown even in the absence of diabetes and hyperglycemia[4, 5]. Clinical evidence to support exogenous induced hyperinsulinemia's role to increase CVD risk is still uncertain [6]. Recent clinical studies suggest exogenous treatment with long acting insulin did not increase CVD events; yet, other studies indicated that improved glycemic control with insulin in type 2 diabetes (T2D) did not reduce CVD events[7–9].

Experimentally, insulin at physiological and hyperinsulinemic levels can induce many actions on the vasculature which may have both anti- and pro-atherogenic effects[10, 11]. Insulin's potential anti-atherogenic effects include activating endothelial NOS (eNOS) and regulating the expressions of vascular adhesion molecule-1[12] (VCAM-1) and antioxidant enzymes heme oxygenase-1 (HO-1)[12–14]. The pro-atherogenic actions of insulin may involve the expressions of PAI-1, endothelin-1 and the migrations and proliferation of smooth muscle cells[15–17]. Previously, we reported that inhibition of insulin's actions by deleting insulin receptors on the endothelial cells accelerated atherosclerosis whereas its enhancement with IRS-1 expression prevented or delayed the development of atherosclerosis[12, 18]. Further, the induction of endogenous hyperinsulinemia in a mouse model of heterogeneous global deletion of insulin receptor did not alter the atherosclerotic process [19]. The present study characterized the effect of exogenous insulin to induce hyperinsulinemia and determined its effect in a mouse model of atherosclerosis. Further, we determined whether the normalization of hyperglycemia alone, using sodium glucose co-transporters 1 and 2 (SGLT(1/2)) inhibitor, in the presence of endogenous hyperinsulinemia, can also affect the atherosclerotic process.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Effect of exogenous hyperinsulinemia on atherosclerosis in Apolipoprotein E deficient mice (ApoE$^{-/-}$) mice on high fat diet (HFD)

Consistant with previous reports[18], both sham treated (ApoE$^{-/-}$) and insulin-implanted ApoE$^{-/-}$ mice, fed on HFD for 16 wks, increased body weights greater than RD-control mice (1.8 fold), similarly, ($p<0.01$, Fig. 1A). Systemic insulin resistance as assessed by IPITT, were elevated equally by HFD in ApoE$^{-/-}$ or insulin implanted ApoE$^{-/-}$ mice compared to RD fed mice (Fig. 1B, $p<0.05$). No differences were observed between sham-control and insulin-implanted ApoE$^{-/-}$ mice on HFD in fat distribution which was increased vs. RD fed mice (Suppl. Fig. I. A). However, insulin-implanted HFD ApoE$^{-/-}$ mice exhibited significant lowered fasting plasma glucose (Suppl. Fig. I. B) (87mg/dL vs. 205mg/dL), respectively, elevated fasting plasma insulin (2.6 fold) and IGF-1 levels (2.7 fold), compared to control ApoE$^{-/-}$ mice fed on HFD at 16 weeks (Fig. 1C and Suppl. Fig. I. C). However, insulin-treated HFD ApoE$^{-/-}$ mice showed reduction in plasma cholesterol levels by 33% ($p<0.05$), but had similar triglyceride levels (TG) compared to ApoE$^{-/-}$ mice on HFD (Suppl. Fig. II).

The severity and complexity of atherosclerotic lesions in the aortic roots and descending aorta were analyzed by multiple methods[20]. After 16 weeks Sudan IV en face staining in HFD sham-treated mice was increased by 6.7 fold ($p<0.001$), (Fig. 1D) and by 5 fold ($p<0.0001$) in insulin-implanted HFD vs. RD fed controls. However, the extent of fatty lesions were decreased by 20% ($p<0.05$) in insulin-implanted, compared with sham-treated HFD-fed ApoE$^{-/-}$ mice (Fig. 1D). Analysis of serial sections throughout the aortic root showed that insulin-implanted ApoE$^{-/-}$ mice exhibited a significant reduction in lesion area stained for collagen (Trichrome) (15%, $p<0.05$), and macrophage content (MAC2) (26%, $p<0.05$) compared to sham-treated ApoE$^{-/-}$ mice on HFD (Fig. 1E and F) but no difference was observed in SMC content. The protein expression of VCAM-1 levels a marker for atherosclerosis and down regulated by insulin[12, 1821] were increased in the aorta of HFD-ApoE$^{-/-}$ mice by 307±47% vs. RD fed mice ($p<0.01$, Fig 1G), which was reduced in the insulin-implanted ApoE$^{-/-}$ mice by 37% ($p<0.01$) vs. HFD-sham ApoE$^{-/-}$ mice (Fig. 1H).

Effect of lowering glucose levels by phlorizin

To determine the effect of lowering plasma glucose on the extent of atherosclerosis, the action of sodium glucose co-transporters inhibitor (SGLT (1/2)), phlorizin were studied (Suppl. Fig. III). Glycated hemoglobin (HbA1c) and albumin levels in the plasma from HFD-ApoE$^{-/-}$ mice (Fig. 2A) were decreased by treatments with insulin or phlorizin equally ($p<0.05$) compared to HFD sham-treated mice, almost to the levels of RD mice. However, urinary glucose was increased only in phlorizin-treated ApoE$^{-/-}$ mice, compared to other groups of ApoE$^{-/-}$ mice fed on HFD (Fig. 2B). There were no differences in the body weights between phlorizin-treated or untreated HFD-ApoE$^{-/-}$ mice, although insulin-treated ApoE$^{-/-}$ mice weighed slightly more than other two groups of mice (Suppl. Fig. IV). IP-GTT showed HFD treatment increased plasma glucose to > 400mg/dL at 30 min indicating mild diabetes. The incremental area under the curve and plasma glucose peak levels from insulin-implanted and phlorizin-treated ApoE$^{-/-}$ mice at both 1 and 2 hr were half as those

in the HFD-sham-treated ApoE$^{-/-}$ mice (P < 0.001) (Fig. 2C). After adding either sham or phlorizin in drinking water 8 weeks after the initiation of HFD, the extent of the atherosclerosis by *en face* assessment, macrophage and collagen content and plaque necrosis in the aortic root did not differ in HFD- ApoE$^{-/-}$ mice (Fig. 2D–G). However the severity of atherosclerosis and necrosis were significantly decreased in the insulin-treated ApoE$^{-/-}$ mice (p=0.039, p=0.017, respectively; Fig. 2D and 2F) comparing to sham-treated HFD-ApoE$^{-/-}$ mice with and without phlorizin. However, macrophage proliferation (Ki67 staining) and apoptosis (TUNEL) were not changed by insulin treatment in the aortic wall vs. other conditions (Suppl. Fig. V. C and D)

Effect on plasma lipoproteins and inflammatory cytokines

Changes in lipoprotein levels were studied[18, 22] and no significant difference in total plasma cholesterol and TG levels between phlorizin-treated or sham-treated HFD-ApoE$^{-/-}$ mice was observed. However, insulin-implanted ApoE$^{-/-}$ mice exhibited reduced total plasma cholesterol by 43% (p<0.05) but not in TG levels (Fig. 3A and B). Lipoprotein profile did not differ between phlorizin-treated or sham-treated HFD-ApoE$^{-/-}$ mice. Insulin-implanted ApoE$^{-/-}$ mice exhibited greater elevation of HDL and diminished levels of VLDL and LDL (Fig. 3C, Suppl. Fig. VI. A and B). Further, LPL activity relative to plasma volume were significantly higher in insulin-implanted ApoE$^{-/-}$ mice than phlorizin-treated or sham-treated HFD-ApoE$^{-/-}$ mice (P < 0.05; Suppl. Fig. VII. A and B). In subcutaneous white adipose tissue (scWAT) and liver, exogenous insulin treatment increased the level of *Lpl* mRNA, which were not altered by phlorizin-treated or sham-treated HFD-ApoE$^{-/-}$ mice (Suppl. Fig. VII. C and D).

Proinflammatory cytokines, interleukin 6 (IL6), and tumor necrosis factor alpha (TNFα) levels in the plasma and aorta were reduced in insulin-implanted ApoE$^{-/-}$ mice by 42%, and 38%, respectively (P<0.001) compared with sham-treated ApoE$^{-/-}$ mice [23–25]. Phlorizin treated HFD-ApoE$^{-/-}$ mice did reduce plasma IL6 and TNFα levels although their levels trended lower (Fig. 3D and E). Expression of *Il6, Tnfα* and *Vcam-1* mRNA levels in the aortic roots were also elevated in HFD sham-treated ApoE$^{-/-}$ mice compared to RD fed mice, but they were not reduced in phlorizin-treated HFD-ApoE$^{-/-}$ mice (Fig. 3F–G). Interestingly, Akt activation (pAkt) were significantly increased in insulin-implanted vs. HFD sham-treated and phlorizin treated ApoE$^{-/-}$ mice (Fig. 3H and I) (p<0.01, p<0.01 & p<0.01) respectively. Since nitric oxide (NO) can be induced by insulin and pAkt[26], we assessed the activation of eNOS in the aorta, which were increased in insulin-implanted ApoE$^{-/-}$ compared to phlorizin-treated and sham-treated HFD ApoE$^{-/-}$ mice (Fig. 3J and K) (p<0.01).

Changes in circulating and tissue monocytes and macrophages polarization

Similar to previous reports, [27–30] circulating Ly6C^{+}/CCR2^{+} monocytes, as measured by fluorescence-activated cell sorting (FACS) analysis, were significantly increased by 34% in HFD-sham-treated compared with RD-ApoE$^{-/-}$ mice (p<0.01). However, there were no differences in circulating Ly6C^{+}/CCR2^{+} monocyte levels amongst HFD-ApoE$^{-/-}$ mice with sham, phlorizin and insulin treatments (Suppl. Fig. VIII). FACS analysis of isolated aortic cells showed that the levels of inflammatory aortic macrophages (F4/80+/CD45+) were

significantly elevated by 3.7 fold ($p<0.01$) in HFD-sham-treated vs. RD-ApoE$^{-/-}$ mice, which were decreased by 47% in insulin implanted ApoE$^{-/-}$ mice ($p<0.01$, Fig. 4A). No differences in macrophages were noted between phlorizin-treated and sham-treated HFD-ApoE$^{-/-}$ mice (Fig. 4A). FACS analysis of the proportion of macrophages exhibiting pro-inflammatory M1 (CD206^{-}/CD80^{+}) or anti-inflammatory M2 (CD206^{+}/CD80^{-}) cell surface markers showed HFD increased M1 pro-inflammatory macrophages and decreased M2 anti-inflammatory macrophages in the aorta. In contrast, exogenous insulin treatment shifted the macrophage population away from M1 towards M2 subtype, but phlorizin was ineffective (Fig. 4B). In addition, insulin treatment decreased the expression of *Ccr7*, *Cxcl10* and *iNos* in M1 macrophages (Fig. 4C) and increased M2 related genes such as *Arg1*, *Ccl17* and *Cd206* (Fig. 4D). To identify the source of circulating TNFα and IL6, gene expression of *Tnfα* and *Il6* in white adipose tissue (WAT) and brown adipose tissues from the HFD-ApoE$^{-/-}$ mice were studied. *Tnfα* and *Il6* mRNA expressions in subcutaneous WAT (scWAT) was inhibited by exogenous insulin treatment but not by phlorizin treatment (Suppl. Fig. IX). In addition, MAC2-DAB^{+} and F4/80^{+} immunostaining macrophages (M1) were abundant in scWAT from both HFD-ApoE$^{-/-}$ mice in the absence or presence of phlorizin treatment but they were decreased in insulin-treated HFD-ApoE$^{-/-}$ mice (Fig. 4E). Flow cytometry showed that M1 subtypes were reduced and M2 subtypes were increased in stromal vascular fraction (SVF) cells isolated from exogenous insulin treated HFD-ApoE$^{-/-}$ mice compared to other groups of mice (Fig. 4F).

Endothelial function and eNOS/NO signaling on M1/M2 polarization

To determine directly whether NO can affect M1/M2, nitric oxide donor, S-Nitrosoglutathione (GSNO) was added to THP-1 cells in the presence and absence of LPS and interferon-γ(IFN-γ). FACS analysis showed that LPS/IFN-γ treatment increased CD80^{+}/CD206^{-} sub-population, which were inhibited only by GSNO, while insulin or phlorizin's have no effect (Fig. 5A–C). Moreover, qRT-PCR analysis of pro-inflammatory genes (*Tnfα* and *Il6*) and M1 polarized genes (*iNos* and *Ccr7*) expression also supported that GSNO inhibits LPS/IFN-γ mediated inflammation and increased in M2 polarization (Fig. 5D and E). To explore the relationship between endothelial eNOS/NO signaling and changing M1 and M2 phenotype, we co-cultured primary lung endothelial cells from ECIRS1 mice which exhibited increased Akt/eNOS signaling and enhanced NO production with THP-1 cells. The induction of M1 (CD80^{+}/CD206^{-}) was decreased and M2 (CD80^{-}/CD206^{+}) was enhanced in EC from ECIRS1 mice after insulin stimulation compared to control EC (Fig. 5F–H). However, the addition of an inhibitor of eNOS activities, $_{L}$-NAME attenuated the decrease M1 and increase in M2 polarization, suggesting that enhancing eNOS/NO activity in EC can decrease M1/M2 ratio in the aorta (Fig. 5F–H).

Discussion

Hyperinsulinemia due to either endogenous origin such as insulin resistance or exogenous sources as in insulin therapy, has been implicated clinically to enhance the development of atherosclerosis[5, 31, 32]. Previously we have reported that the severity of atherosclerosis was not affected by endogenously induced hyperinsulinemia in the absence of systemic insulin resistance[19]. In contrast, we reported here that the effect of exogenously applied insulin

decreased the severity of atherosclerosis in ApoE$^{-/-}$ mice on HFD which generated hyperinsulinemia, insulin resistance and hyperglycemia. Unlike the endogenously generated hyperinsulinemic ApoE$^{-/-}$ model, the present study was performed in ApoE$^{-/-}$ mice on HFD which induced weight gain, insulin resistance, elevated levels of abdominal and visceral fat, hyperglycemia and hyperinsulinemia, which were elevated further (2x) by insulin implants. The implanted insulin improved glucose tolerance tests and significantly increased IGF1 levels, but did not affect weight gain or fat mass. The inability of endogenously derived hyperinsulinemia to affect the atherosclerosis in ApoE$^{-/-}$ mice with IR partial deletion, as reported previously, suggests that doubling of plasma insulin levels were not adequate to affect the atherosclerotic process[19]. The elevation of IGF-1 levels was unlikely to affect the atherosclerotic process since the IGF-1 levels attained by insulin treatment only returned them to normal and physiological levels.

Since multiple metabolic parameters were changed with the insulin implant, due to the metabolic actions of hyperinsulinemia, the explanation for the decrease in atherosclerosis is likely to be complex. Major differences induced by the exogenous insulin implants compared to endogenous induced hyperinsulinemia were lowering of plasma glucose, lipoproteins and inflammatory markers. To test whether the singular effect of glycemic control by implanting insulin in this insulin resistant and diabetic model can affect atherosclerosis, glycemic control was improved using phlorizin, a selective inhibitor of SGLT(1/2), which enhanced glycosuria, decreased HbA1c and glycated albumin, without changes in lipoprotein profile[33, 34]. Interestingly, lowering plasma glucose alone, did not affect the severity of atherosclerosis in the HFD-fed ApoE$^{-/-}$ mice model. These results suggest that normalizing plasma glucose alone without decreasing fat composition and altering inflammatory process was inadequate to affect the atherosclerosis process in insulin resistance and diabetes. These findings in the ApoE$^{-/-}$ mice are consistent with clinical trials which have shown glycemic control alone may not decrease cardiovascular disease (CVD) in people with T2D[2, 7–9]. Our study supports Yoon et al who showed that chronic insulin treatment has beneficial effects on dyslipidaemia, IL6 levels and atherosclerosis in *Ldlr*$^{-/-}$ mice with diet-induced obesity and diabetes[35].

Recently, EMPA-REG Outcome study reported that SGLT2 inhibitor treatment reduced cardiovascular mortality and heart failure in patients with T2D, although the incidence of fatal/nonfatal myocardial infarction and nonfatal stroke were not changed. This indicates that the beneficial effect of SGLT2 inhibitor was driven by improvement in the myocardial function, which occurred after a few months of treatments[36, 37] rather than reversing atherosclerotic lesion, which will require years to occur[38]. Contrary to our study, Nagareddy et al[39] has reported that lowering glucose levels by SGLT2 inhibitor decreased diabetes-induced monocytosis and myelopoiesis in STZ-induced diabetic and Akita mice and decreased atherosclerotic lesions in STZ-induced *Ldlr*$^{-/-}$ mice. However, their study differed significantly from our study, since streptozotocin or Akita induced diabetes are insulin deficient models whereas our ApoE$^{-/-}$ mice have hyperinsulinemia and mild diabetes due high fat diet (60%), which mimics T2D[40].

Another potential anti-atherosclerotic effect of hyperinsulinemia in the HFD fed ApoE$^{-/-}$ mice could be due to improvement in lipoprotein profile. Insulin implant significantly

lowered VLDL and LDL levels and decreased atherosclerosis in ApoE$^{-/-}$ mice[41]. Interestingly, the levels of atherosclerosis in insulin-treated ApoE$^{-/-}$ mice, was less than those in ApoE$^{-/-}$ mice fed Western diet (WD, 42% of Kcal) although there were no differences in plasma cholesterol levels between these two ApoE$^{-/-}$ models (Suppl. Fig. X). Exogenous insulin implant also significantly reduced various inflammatory circulating cytokines and their expression on the aorta including IL6, TNFα and VCAM-1. Besides the reduction of inflammatory cytokines, our results also suggested that exogenously-treated hyperinsulinemia decreased macrophages in the aorta, but did not affect systemic monocytosis induced by HFD. The improvements of insulin's anti-inflammatory actions are likely to be mediated via several mechanisms such as decreasing VCAM-1 expression, activation of eNOS in the endothelial cells and altering the M1/M2 ratio toward the anti-inflammatory profile.

In summary, this report demonstrated that exogenously induced hyperinsulinemia, even in the presence of insulin resistance, hyperglycemia and hyperlipidemia, can decrease the severity and complexity of the atherosclerotic process. The anti-atherogenic effect of the exogenously induced hyperinsulinemia is not due to the lowering of plasma glucose alone, but is also related to decreasing LDL and VLDL levels and reductions of inflammatory cytokines. The effect of insulin on improving lipid profile is probably related to its actions in the liver and the activation of lipoprotein lipase as previously reported[42, 43]. However, insulin also had significant anti-inflammatory actions of inhibiting VCAM-1 and activities eNOS in the endothelium to decrease monocyte uptake into the vascular wall and the expression of the various inflammatory actions. Clinical significance of these findings in rodent models of atherosclerosis and insulin resistance will need to be evaluated further.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

CVD	Cardiovascular diseases
HFD	High Fat Diet
ApoE$^{-/-}$ mice	Apolipoprotein E deficient mice
SGLT (1/2)	Sodium glucose co-transporters inhibitor (1/2)
eNOS	Endothelial NOS
VCAM-1	Vascular adhesion molecule-1

TNFα	Tumor necrosis factor alpha
IL6	Interleukin 6

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Highlights

- Lowering glucose using SGLT(1/2) inhibitor did not affect the severity of atherosclerosis in the fed ApoE$^{-/-}$ mice.
- Insulin-implanted ApoE$^{-/-}$ mice exhibited greater elevation of HDL and diminished levels of VLDL and LDL.
- Exogenous insulin treatment has no effect on circulating monocytes (CCR2/Ly6C) but decreases inflammatory macrophages (F4/80/CD45) in aorta of ApoE$^{-/-}$ mice fed an HFD.
- Improved endothelial function through eNOS/NO activations reduced pro-inflammatory (M1) and increased anti-inflammatory (M2) macrophages

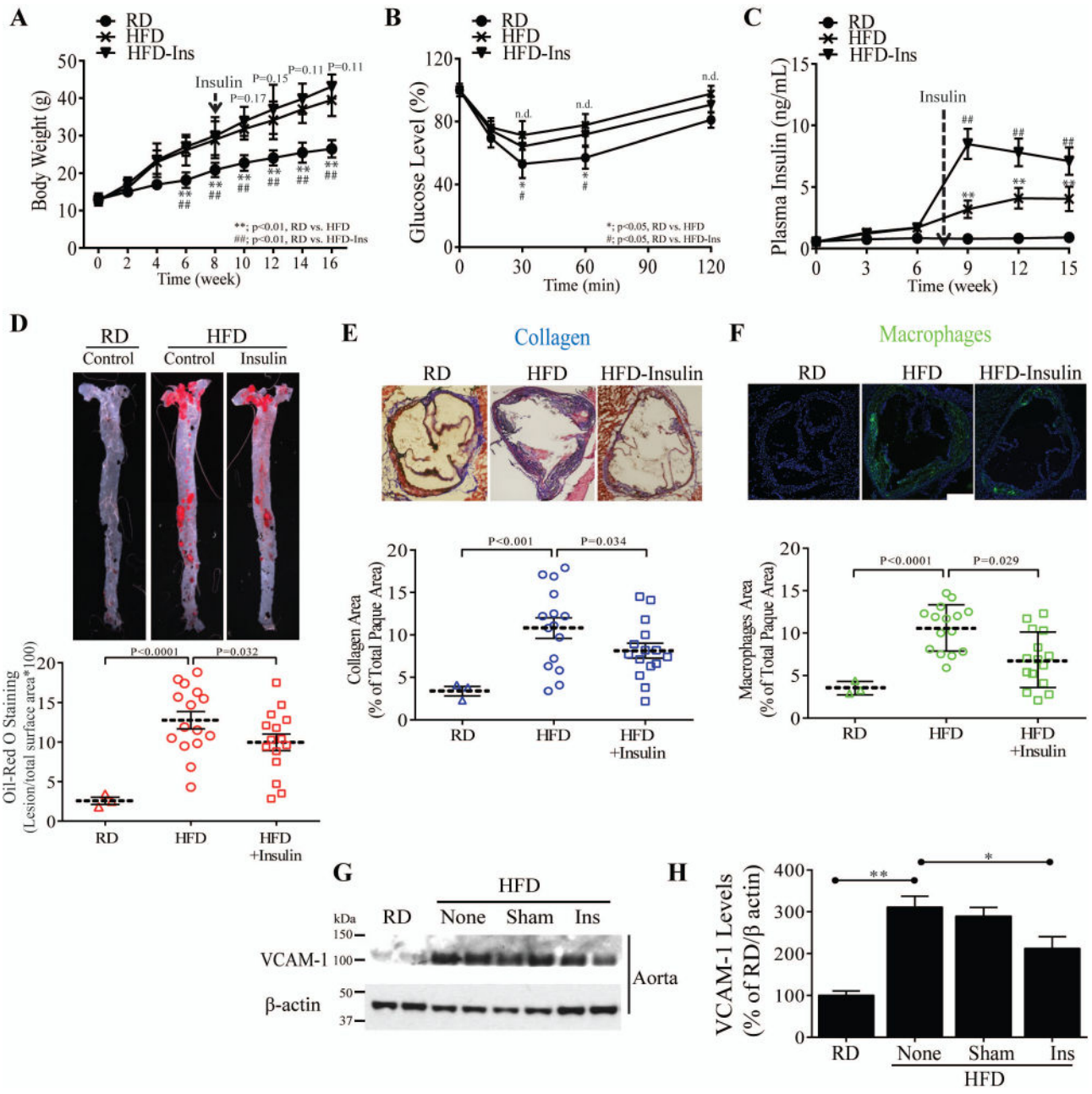

Figure 1. Effect of exogenous insulin implant-induced hyperinsulinemia on atherosclerosis and its complexity in the ApoE$^{-/-}$ mice fed on HFD

(A) Body weights of experimental animals during HFD feeding (n = 9 for control, n = 9 for insulin treatment). (B) Insulin sensitivity (IPITT) (C) Time course of plasma fasting insulin. (D) *en face* staining and quantification of aortas as percent of lesion area without or with insulin treatment (n = 10 per group). (E and F) Representative examples and quantification of cross-sections from the aortic sinus stained with trichrome (E) and MAC2(F). (G and H) Immunoblot and densitometry of VCAM-1 levels in aorta from control, insulin treated and phlorizin-treated ApoE$^{-/-}$ mice fed on HFD. All data are presented as mean ± SEM. *p < 0.05, **p < 0.01. P-values for panels A, B, and C were computed using linear mixed effects

models to account for the repeated measures within animals over time. P-values for panels A, B, and C were computed using linear mixed effects models to account for the repeated measures within animals over time. P-values for panels D, E, F, and H were computed using general linear model (ANOVA). In all cases, post hoc t-tests with Tukey's adjustment for multiple comparisons were conducted where overall F-tests were significant to ascertain the location of any significant pairwise differences.

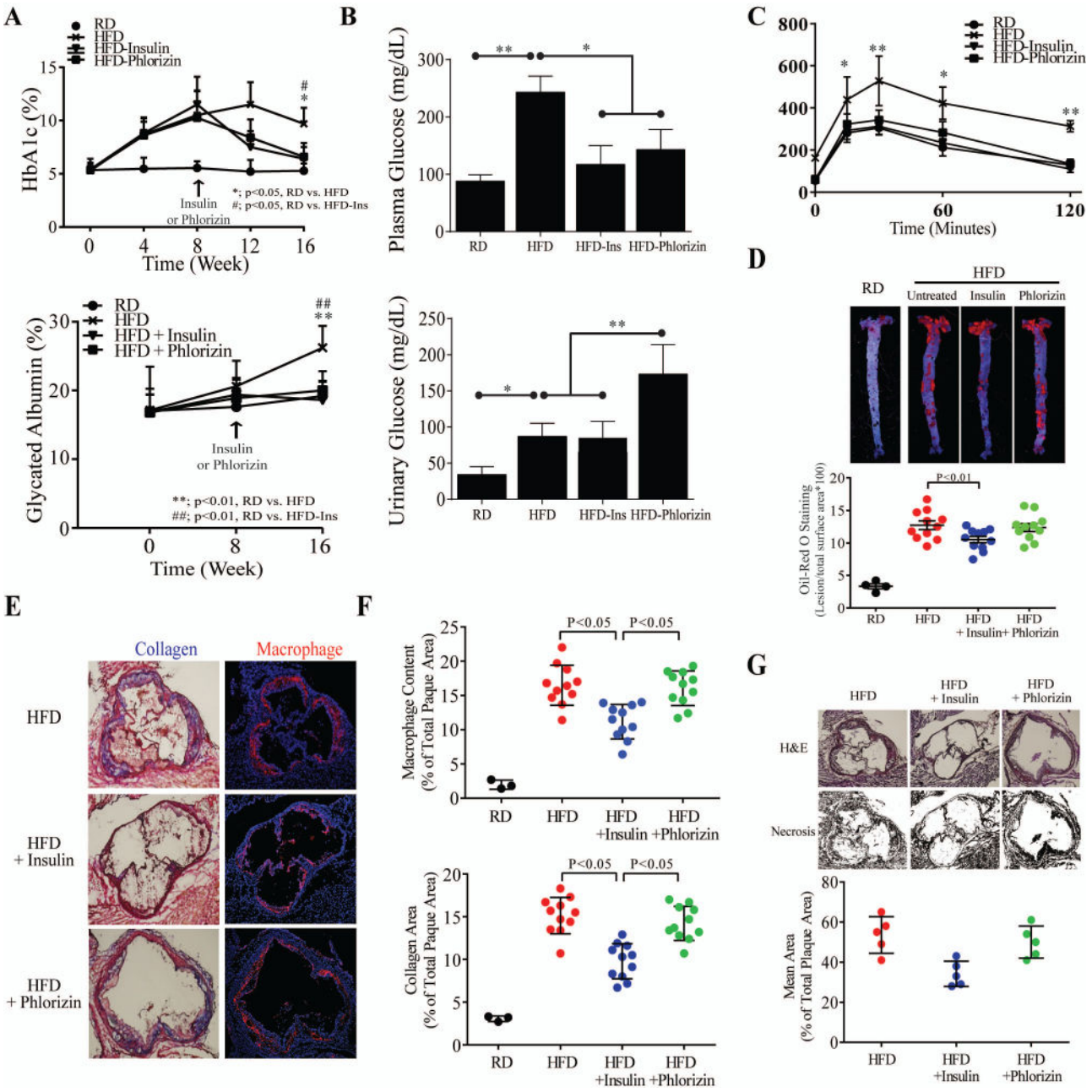

Figure 2. Effect of lowering HFD-induced plasma glucose levels on atherosclerosis

(A) Time-response curves for inhibitory effects of phlorizin on plasma HbA1c (Upper) and glycated albumin (Lower) from ApoE$^{-/-}$ and insulin treated ApoE$^{-/-}$ mice with feeding HFD. (B) Fasting plasma and urinary glucose measurements from control and insulin or phlorizin treated HFD-ApoE$^{-/-}$ mice. (C) Glucose tolerance test (IPGTT) (n = 8 for control, insulin treatment and phlorizin treatment). (D) Oil Red O staining (Upper) and quantification (Lower) of aortas as percent of lesion area from the same condition as A-C (n = 11 per group). (E) Quantification of cross-sections from the aortic sinus stained for collagen (Upper) and macrophages (Lower). (F) Representative sections of hematoxylin and eosin-stained aortic root sections and quantification of necrotic area in a subgroup of 6

control lesions, insulin-treated lesions and phlorizin-treated lesions with statistically identical lesion area. All data are presented as mean ± SEM. *$p < 0.05$, **$p < 0.01$. P-values for panels A and C were computed using linear mixed effects models to account for the repeated measures within animals over time. P-values for panels B, D, E, F, and G were computed using general linear model (ANOVA). In all cases, post hoc t-tests with Tukey's adjustment for multiple comparisons were conducted where overall F-tests were significant to ascertain the location of any significant pairwise differences.

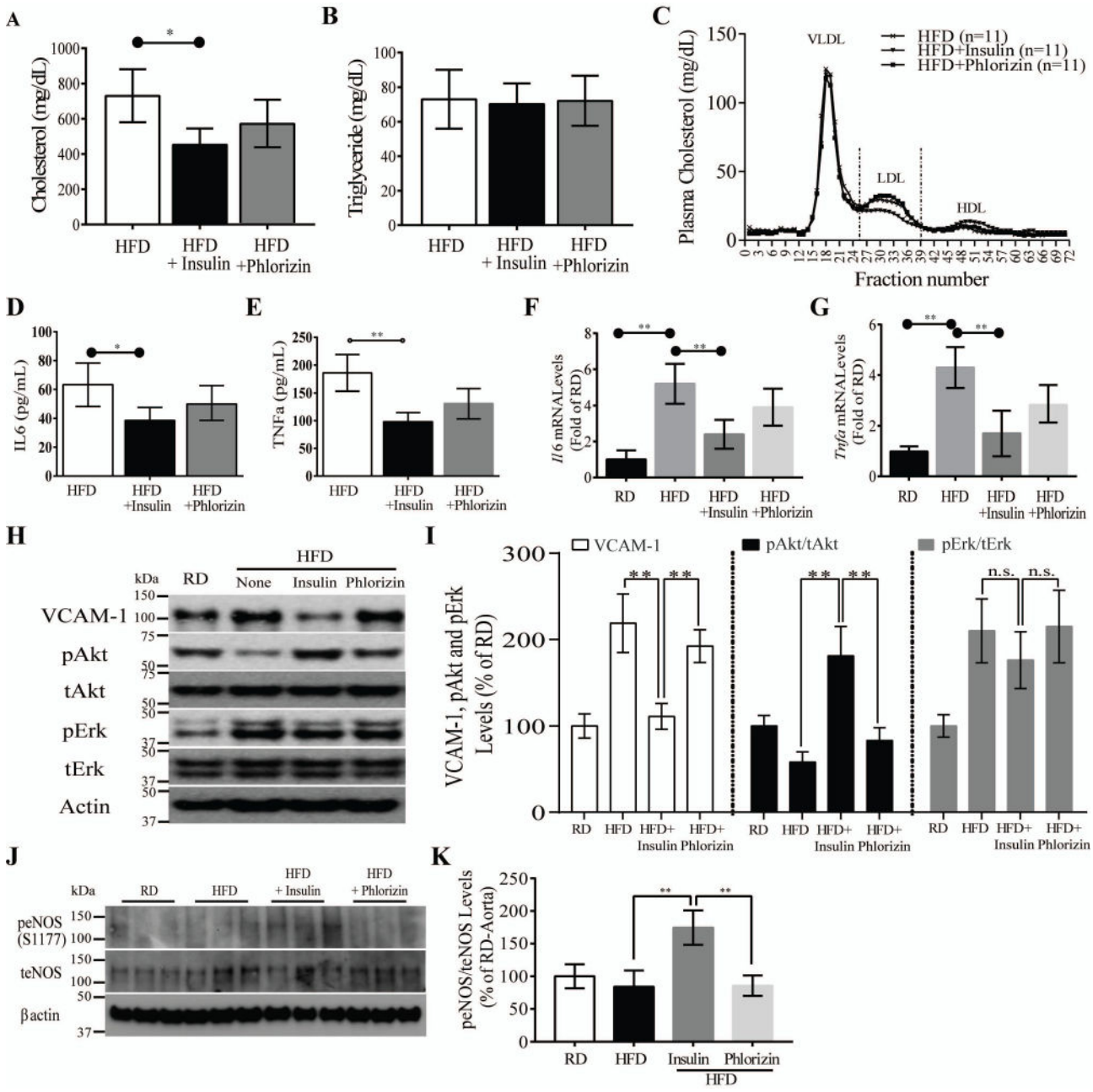

Figure 3. Effect of exogenous insulin and phlorizin on lipoprotein and inflammatory cytokine levels in plasma of HFD-ApoE$^{-/-}$ mice

(A and B) Total plasma cholesterol (A) and triglycerides (B) of HFD-fed control, insulin treated and phlorizin-treated ApoE$^{-/-}$ mice (n = 6, respectively). (C) Pooled plasma samples were subjected to fast performance liquid chromatography gel-filtration, and the fractions were assayed for cholesterol concentration. (D and E) TNF-α (D) and IL6 (E) plasma levels from all ApoE$^{-/-}$ mice. (F and G) The gene expressions of the inflammatory markers *Il6* mRNA (F) and *Tnfα* mRNA (G) were measured in pooled aortic arch from all ApoE$^{-/-}$ mice. (H and I) Immunoblot and densitometry of Akt and Erk activity, and VCAM-1 levels in aorta from all ApoE$^{-/-}$ mice. (J and K) Immunoblot and densitometry of eNOS activity in

aorta from all ApoE$^{-/-}$ mice. All data are presented as mean ± SEM and representative of three independent experiments. *$p < 0.05$, **$p < 0.01$. P-values were computed using general linear model (ANOVA). Post hoc t-tests with Tukey's adjustment for multiple comparisons were conducted where overall F-tests were significant to ascertain the location of any significant pairwise differences.

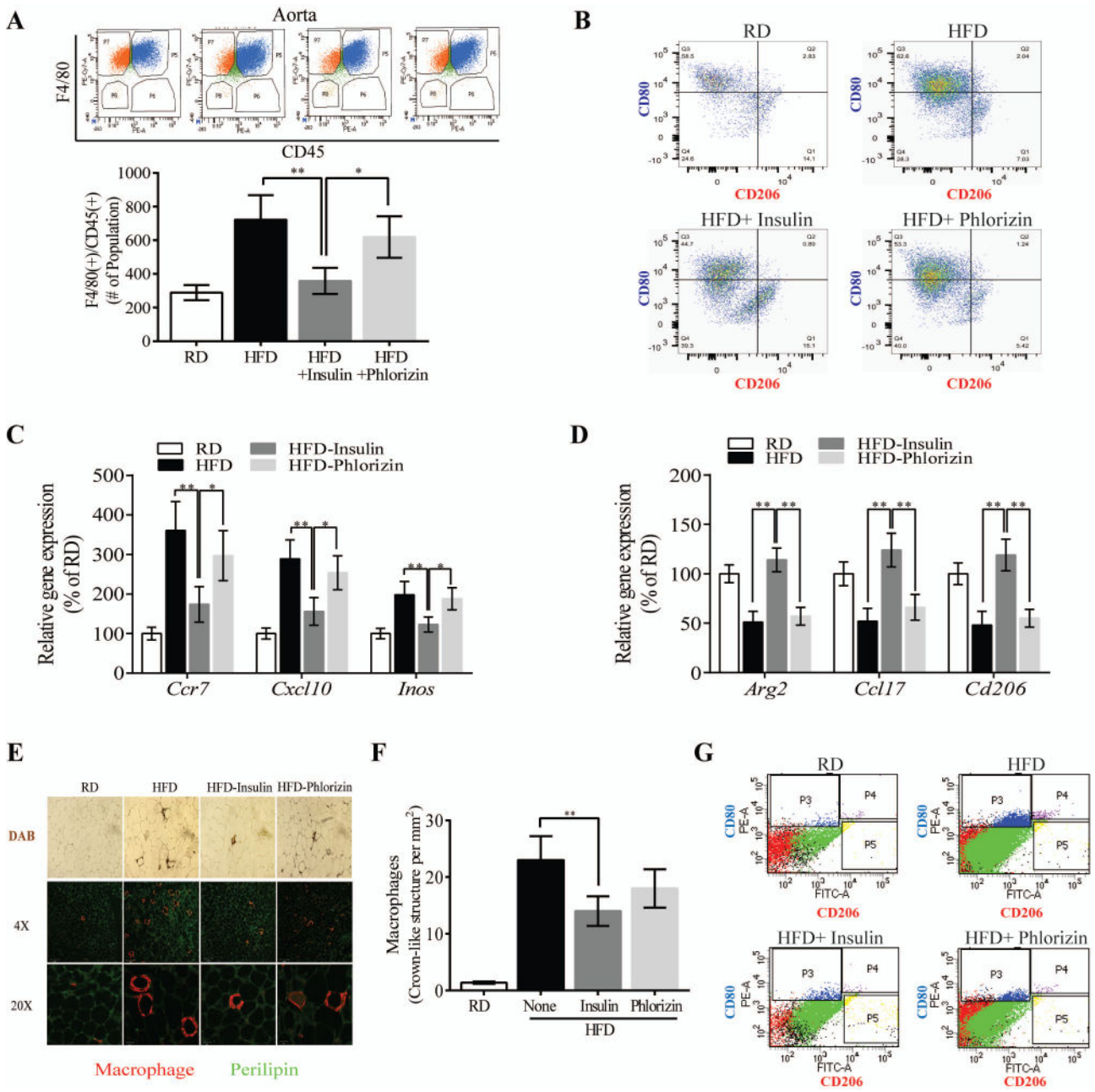

Figure 4. Effect of exogenous insulin and phlorizin on HFD-induced macrophge plasticity in aorta and subcutaneous white adipose tissue

(A) Total number of retrieved F4/80(+)/CD45(+) inflammatory macrophages per aorta in ApoE null mice fed on either RD or HFD in combination with insulin and phlorizin. (B) FACS analysis shows staining of CD80 (M1) or CD206 (M2) in aorta from the mice. Panels show dot plot, and representative percentages indicate each subset as a proportion of total F4/80+/CD11b+/CD45+ macrophages. (C and D) M1 (*Ccr7, Cxcl10, and iNos*) and M2 (*Arg1, Ccl17, and Cd206*) genes expression in arota. Data show % in gene expression by RT-qPCR. (E) DAB and immunostaining of adipose tissue macrophages in scWAT. (F) FACS analysis shows staining of CD80 or CD206 in the SVF of scWAT from these mice.

Results are pooled from 3 independent experiments with 2–5 mice per group. All data are presented as mean ± SEM and representative of three independent experiments. *$p < 0.05$, **$p < 0.01$. P-values were computed using general linear model (ANOVA). Post hoc t-tests with Tukey's adjustment for multiple comparisons were conducted where overall F-tests were significant to ascertain the location of any significant pairwise differences.

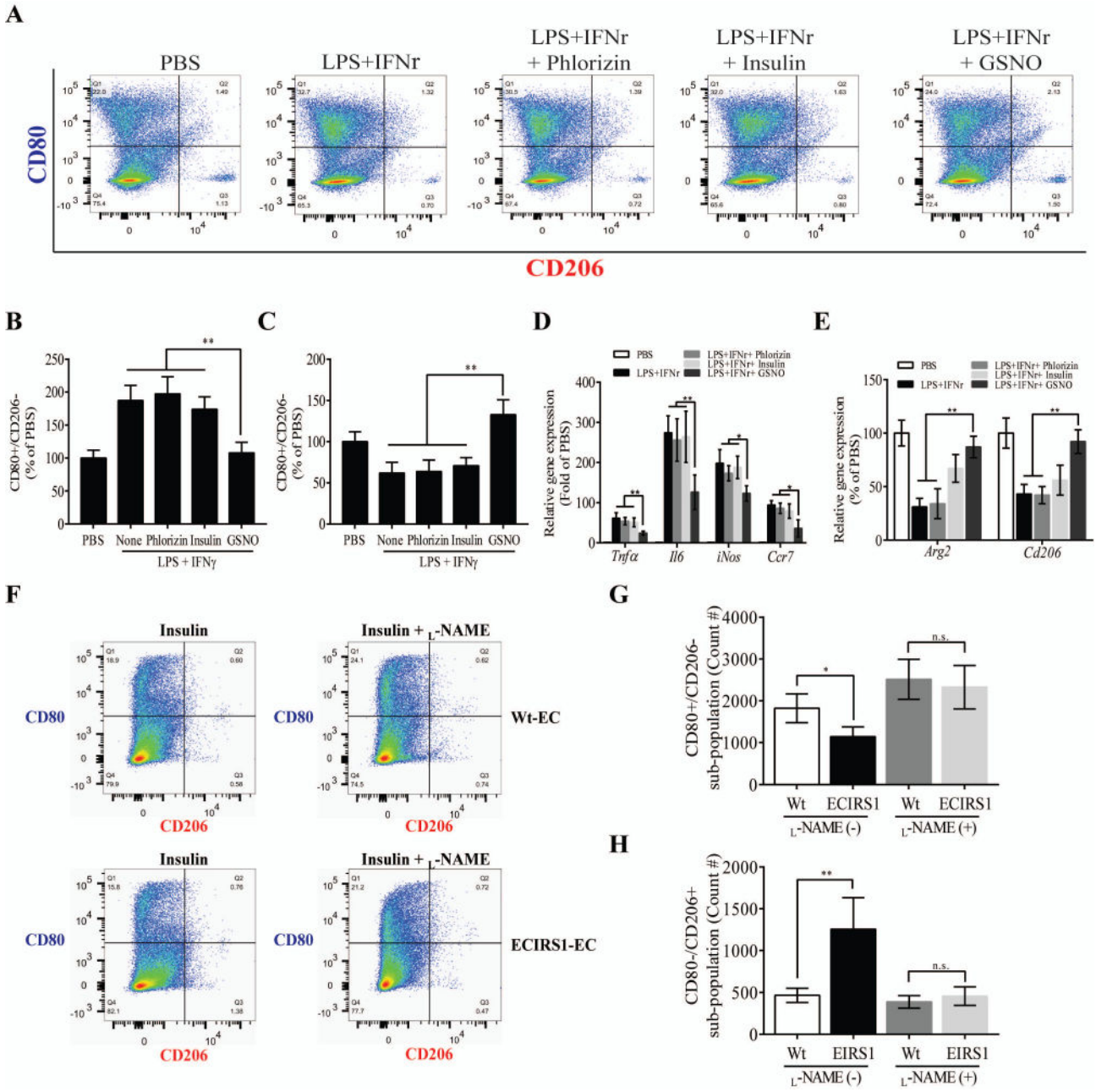

Figure 5. Effect of endothelial function via NO production on M1 and M2 macrophagy polarization *in vitro*

(A) FACS analysis shows staining of CD80 (M1) or CD206 (M2) in THP-1 cells stimulated by LPS and IFNr with Phlorizin, insulin, or GSNO. (B and C) Quantification of FACS analysis for CD80+/CD206- (M1) and CD80−/CD206+ (M2) in THP-1 cells (A). (D) Inflammatory (*Tnfα* and *Il6*) and polarized M1 (*Ccr7*, *Cxcl10*, and *iNos*) gene in THP-1 cells under the same condition as (A). (E) Polarized M2 (*Arg1*, *Ccl17*, and *Cd206*) genes expression in THP-1. (F) THP-1 cells were cocultured with EC from either Wt or ECIRS1 mice, stimulated with insulin in the absence or presence of L-NAME treatment. (G and H) Quantification of FACS analysis for CD80+/CD206- (M1) and CD80−/CD206+ (M2). All

data are presented as mean ± SEM and representative of three independent experiments. *p < 0.05, **p < 0.01. P-values were computed using general linear model (ANOVA). Post hoc t-tests with Tukey's adjustment for multiple comparisons were conducted where overall F-tests were significant to ascertain the location of any significant pairwise differences.