Supplement Material

Hyperinsulinemia does not change atherosclerosis development in apolipoprotein E null mice.
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Supplementary research design and methods

Animals

Cross-breeding of mice with a "floxed" exon 4 of the insulin receptor (*Insr*) gene, Tie2-cre transgenic mice, and apoE null mice have been described previously [1]. When animals homozygous for both the apoE null mutation and the *Insr* floxed mutations were bred using a cre-positive dam (mother) and a cre-negative sire (father), offspring always had whole-body cre-mediated recombination irrespective of whether they were cre-negative or cre-positive. This recombination was apparent as a higher-mobility band (Δ band) during gel electrophoresis of PCR product where the template was lysate of tail (which contains endothelial, Tie2-expressing cells) or hair (which contains no endothelial cells) [1]. We used female cre-negative, Δ band-positive mothers and cre-negative, Δ band-negative fathers to breed animals used for experiments in the current study (Fig. 1*A*). Littermate controls were used in all analysis comparing $Insr^{flox/\,flox}$ $Apoe^{-/-}$ ("controls") and $Insr^{flox/\Delta}$ $Apoe^{-/-}$ ("haploinsufficient") mice.

The mice had been partly backcrossed to the C57 background, with previous genetic characterization of animals from the same colony showing that animals had 87.6±0.8% of the C57BL/6 background, using an array genotyping 377 single nucleotide polymorphisms [1]. Female mice were used for experiments and fed a regular chow diet with 9.0% (w/w) fat and 0.221 ppm cholesterol (Mouse Diet 9F, LabDiet). All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with NIH guidelines following the standards established by the Animal Welfare Acts and by the documents entitled "Principles for Use of Animals" and "Guide for the Care and Use of Laboratory Animals".

DEXA scanning and food intake

Dual-energy X-ray absorptiometry (DEXA) scanning was performed with animals in general anaesthesia using a PixiMus II instrument (GE Lunar, Madison WI). Food intake was measured over 48 hours while mice were placed in a metabolic cage. The mass of leftover food as well as residual food lost through the grid floor of the cage were subtracted from the mass of food added.

Insulin injection and tissue isolation

Mice were fasted 4 hours before sampling of blood for analysis of plasma lipids and overnight (16 hours) before isolation of tissues. Anaesthesia was induced with pentobarbital. In some animals, insulin, IGF-1, or their vehicle were injected into the vena cava after laparotomy. Blood was drawn by transcutaneous cardiac puncture in a syringe containing EDTA immediately before opening the thorax; in mice injected in the vena cava, blood was drawn by cardiac puncture through the diaphragm. The mouse was then perfused through the left ventricle with a syringe pump set at 1 ml/minute. During perfusion, lung, liver, gastrocnemius muscle, and perigonadal fat were collected immediately and flash frozen in liquid nitrogen, then stored at –70 °C for subsequent analysis of RNA or protein.

If the aorta was to be used for protein analysis, perfusion was completed with 2 ml PBS and the aorta quickly dissected and flash frozen in liquid nitrogen; this entire procedure took <5 minutes. If the aorta was to be used for atherosclerosis quantitation, perfusion was performed with 5 ml of PBS. The ascending aorta was then divided just above the aortic sinus and the base of the heart, and the latter part immediately embedded in OCT medium and frozen. The brachiocephalic artery was dissected free of perivascular tissue and placed in a mixture of chloroform and methanol. While *in situ*, the aorta was dissected free of perivascular tissue, the aortic arch was opened along the minor curvature and the entire aorta opened along the ventral side, then removed and stored in formalin.

Glucose and insulin tolerance tests

Glucose tolerance tests were performed in the morning after an overnight fast. D-glucose 2 mg per gram body weight was injected intraperitoneally. Capillary blood from a tail cut was sampled in heparinized capillary tubes immediately before glucose injection, and 30, 60, and 120 minutes after glucose injection. Insulin tolerance tests were performed in the early afternoon in the random fed state. Insulin (Humulin R, Eli Lilly Companies, Indianapolis, IN) 0.6 mU per gram body weight was injected intraperitoneally and capillary blood sampled from a tail cut.

Insulin clearance

Human insulin with a proline B28 to aspartate mutation, also called insulin aspart (NovoLog, Novo Nordisk) was administered at a dose of 0.5 mU/g as an intravenous bolus in the jugular vein through a catheter placed 3 days previously. Capillary blood from a tail cut was sampled in heparinized capillary tubes immediately before insulin aspart injection and 15, 30, 45, and 60 minutes after injection. Plasma was stored at –70 °C and subsequently analyzed with an ELISA which does not cross-react with native insulin [2]. Accordingly, insulin aspart was not detectable in plasma samples obtained before insulin aspart injection.

Plasma analytes

Glucose was measured in whole capillary blood with an Ascensia Contour blood glucose meter (Bayer HealthCare). Insulin was measured in plasma with ELISA (rat insulin ELISA using mouse insulin as standards, Crystal Chem, Downers Grove, IL) after sampling capillary blood from a tail cut in heparinized capillary tubes. Cholesterol and triglycerides were measured by a colorimetric assay in plasma from blood obtained by cardiac puncture during anesthesia with pentobarbital. These lipids were also measured in plasma fractions obtained by fast protein liquid chromatography.

Western blotting

Frozen tissue was thawed in ice-cold buffer containing Tris (20 mM, pH 7.5), Triton X-100 (1% v/v), sodium pyrophosphate (2.5 mM); NaF (10 mM), NaCl (140 mM), EDTA (1 mM), EGTA (1 mM), β-glycerophosphate (1 mM), Na_3VO_4 (1 mM), leupeptin (10 μg/ml), aprotinin (1 μg/ml), and phenylmethylsulfonylfluoride (1 mM). Tissues were treated with a rotor-stator homogenizer; frozen muscle tissue was powdered before homogenization. Samples were normalized to equal protein concentration, measured by the bicinchoninic acid assay. Whole cell lysate was reduced by addition of modified Laemmli buffer (Invitrogen) and heating to 70 °C for 10 minutes. Samples were then separated on precast minigels (NuPAGE tris-acetate gels, Invitrogen) and electrotransferred to a nitrocellulose membrane. Primary antibodies for Western blotting were

purchased from Cell Signaling Technology (Beverly, MA) with the exception of phospho-tyrosine antibody (Millipore, Billerica, MA), eNOS antibody (BD Biosciences Pharmingen) actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and VCAM-1 antibody (R & D Systems, Minneapolis, MN) and endothelin-1 antibody (Santa Cruz). Densitometry was performed on scanned images of film using ImageJ software (version 1.43u).

Quantitation of atherosclerotic lesion size in the aorta
The whole aorta, from just distal to the aortic sinus to the iliac bifurcation, was used. Residues of perivascular fat were removed under a stereomicroscope. The aorta was then stained for 5 minutes in a filtered solution containing 0.5% Sudan IV, 35% ethanol, and 50% acetone and destained for 5 minutes in 80% ethanol. The stained aortas were placed on a glass slide and cover slipped, placed on a black surface, and photographed (QColor3 Color FireWire 3.3 MP Digital Camera, Olympus) through a stereomicroscope using polarized light. Two exposures covering the length of the aorta were merged in imaging software (Adobe PhotoShop version CS4) and red pixels selected using an identical color tone for all aortas. Lesion area was measured using the Threshold Adjust and Analyze Particles commands in ImageJ and expressed as a fraction of the total surface area of the aorta. In the images shown in Fig. 5, for presentation purposes only, the image area representing the aorta was selected in PhotoShop and placed on a uniformly black background.

Quantitation of lesion size and macrophage content in aortic sinus
The base of the heart with the aortic sinus was embedded in OCT medium and frozen, then cryosectioned in 5 μm sections. Sections were selected for analysis at the level of the sinutubular junction starting with the section where two out of three commissures were first visible when sectioning towards the aorta, and every tenth section selected from this starting point. Sections were stained with hematoxylin and eosin. A fat stain, such as Oil Red O, was not used since we have previously shown that plaques from control mice with the same genetic background contains considerable amount of collagen [1] so that fat-stained area alone would underestimate lesion size. Atherosclerotic cross-sectional area was measured by planimetry using the Magnetic Lasso tool in Photoshop CS4 and the Analyze Particles commands in ImageJ. The average area on at least 3 sections, each separated by 50 μm, was calculated.

Aortic sinus cryosections were also used for immunohistofluorescence. Sections were air dried for 45 minutes and fixed in acetone at -20°C for 10 minutes. Blocking was done sequentially with avidin, biotin (Vector Laboratories, Burlingame, CA), and CAS-Block (Invitrogen). Sections were then incubated with anti-F4/80 antibody (Abcam) at 1:50 dilution followed by biotin-conjugated anti-rat IgG (Vector Labs) and strepavidin conjugated with DyLight 594 (Vector Labs). Photographs taken on a fluorescence microscope were analyzed by ImageJ as described above using the average value from 3 sections.

Analysis of cholesterol content in the brachiocephalic artery
Extracts of the brachiocephalic artery underwent hydrolysis of cholesteryl esters and derivatization of cholesterol followed by liquid chromatography and mass spectrometry. Details of this method have been published recently [1, 3].

Statistical analysis

Responses in glucose and insulin tolerance tests were analyzed by calculating the area under the concentration versus time curve using the linear trapezoidal rule. Analysis of differences between atherosclerotic lesion size was done with Wilcoxon rank-sum test. All other analysis were made using paired or unpaired t-test. Statistical significance was defined as $p<0.05$. In text and graphs, data are presented as the mean ± standard error of the mean.

Supplementary references

1. Rask-Madsen C, Li Q, Freund B, Feather D, Abramov R, Wu IH, Chen K, Yamamoto-Hiraoka J, Goldenbogen J, Sotiropoulos KB, Clermont A, Geraldes P, Dall'Osso C, Wagers AJ, Huang PL, Rekhter M, Scalia R, Kahn CR, King GL. Loss of insulin signaling in vascular endothelial cells accelerates atherosclerosis in apolipoprotein E null mice. Cell Metab 2010;11:379-389.

2. Andersen L, Jorgensen PN, Jensen LB, Walsh D. A new insulin immunoassay specific for the rapid-acting insulin analog, insulin aspart, suitable for bioavailability, bioequivalence, and pharmacokinetic studies. Clin Biochem 2000;33:627-633.

3. Kuo MS, Kalbfleisch JM, Rutherford P, Gifford-Moore D, Huang XD, Christie R, Hui K, Gould K, Rekhter M. Chemical analysis of atherosclerotic plaque cholesterol combined with histology of the same tissue. J Lipid Res 2008;49:1353-1363.

Supplementary figure legends

Supplementary figure I. Genetic modifications and mouse breeding.
Diagram showing generation of *Insr* haploinsufficient mice ($Insr^{flox/\Delta}$ $Apoe^{-/-}$) and littermate controls ($Insr^{flox/flox}$ $Apoe^{-/-}$) by breeding mice with cre-mediated recombination in the germline. Note that both haploinsufficient mice and their controls were cre-negative and had an inherited null mutation in the insulin receptor (*Insr*) gene.

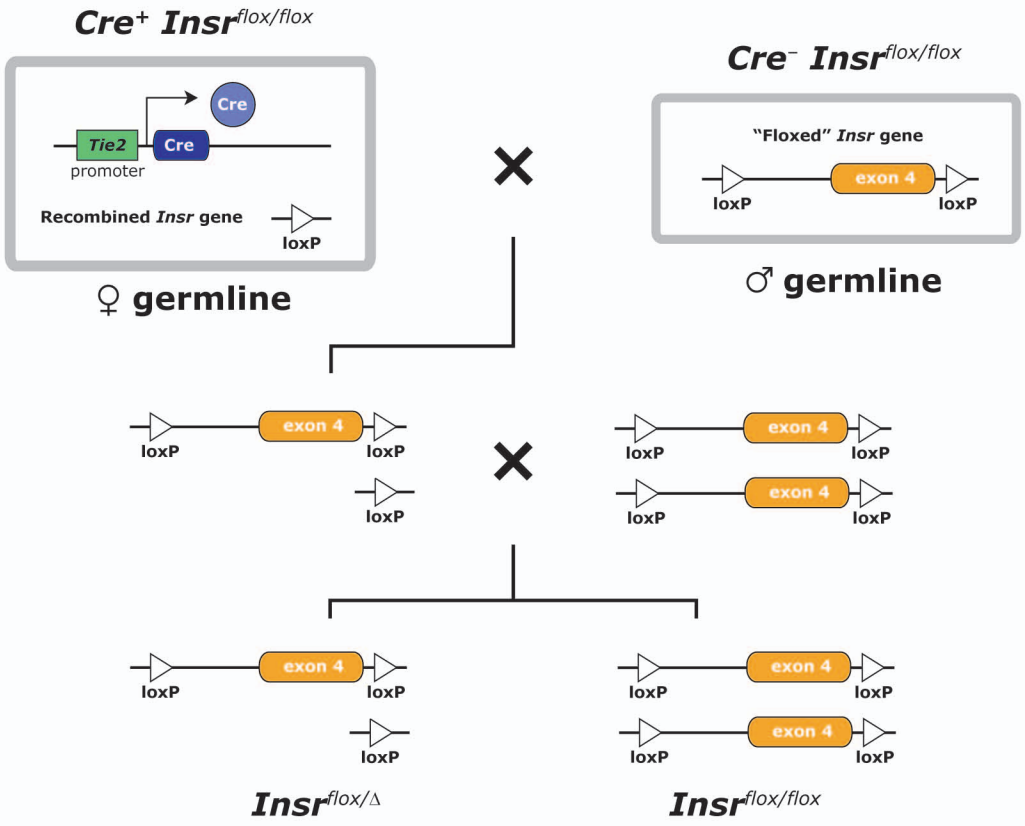
Supplementary figure II. Insulin signaling in muscle, liver and fat.
Insulin at a dose of 0.5 mU/g or insulin diluent was injected in the vena cava during pentobarbital anesthesia. Organs were isolated and flash frozen after 5 minutes. **A**, Representative Western blots based on lysate of gastrocnemius muscle, liver, or fat using tissue from control (c) or haploinsufficient (Δ) mice ($Insr^{flox/flox}$ $Apoe^{-/-}$ and $Insr^{flox/\Delta}$ $Apoe^{-/-}$, respectively). **B**, Average expression values calculated from densitometry of Western blots using tissue from 3 control and 3 haploinsufficient animals.

Supplementary figure III. Plasma lipids.
Serum or EDTA plasma was isolated from blood samples obtained by cardiac puncture during pentobarbital anesthesia after 4 hours fast. **A**, Serum concentrations of free fatty acids in 8 control (c) and 12 haploinsufficient (Δ) mice ($Insr^{flox/flox}$ $Apoe^{-/-}$ and $Insr^{flox/\Delta}$ $Apoe^{-/-}$, respectively). **B**, Plasma concentrations of cholesterol (10 animals in each group) and triglyceride (8 control and 9 haploinsufficient mice). **C**, Cholesterol and triglyceride in fractions from fast protein liquid chromatography (FPLC) of pooled plasma from 10 control and 10 haploinsufficient mice.

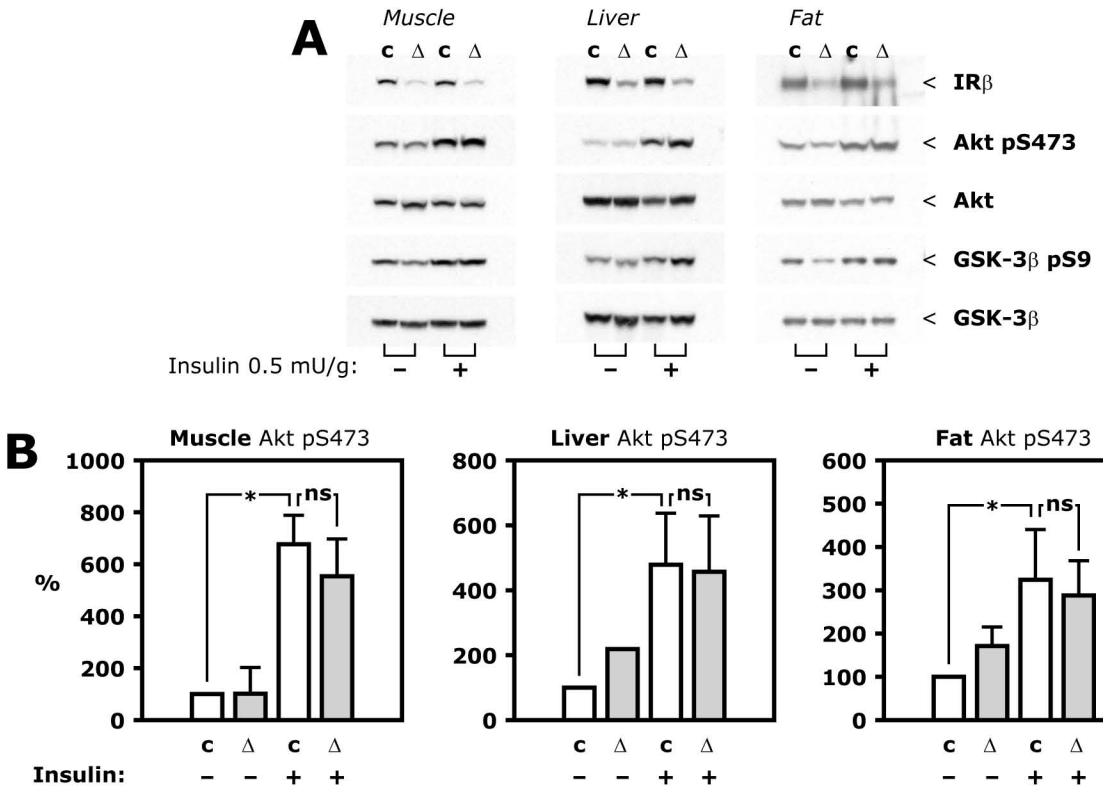
Supplementary figure IV. IGF-1 signaling.
IGF-1 (0.5 μg/g) or PBS was injected into the vena cava during pentobarbital anesthesia. After 5 minutes, the aorta was isolated and flash frozen. Representative Western blots (top) and average expression (bottom) calculated from densitometry of Western blots based on aorta lysate from 4 animals in each group (total of 8 control (c) and 8 haploinsufficient (Δ) mice, $Insr^{flox/flox}$ $Apoe^{-/-}$ and $Insr^{flox/\Delta}$ $Apoe^{-/-}$, respectively).

Supplementary figure V. Macrophage content in aortic sinus plaques and arterial lipid content.
A, Cryosections of aortic sinus were made from tissue isolated from animals at 52 weeks of age. Representative images of immunohistofluorescence of aortic sinus sections stained with antibody towards the macrophage marker F4/80 (red fluorescence) and with the nuclear stain DAPI (blue) using tissue from control (c) and haploinsufficient (Δ) mice ($Insr^{flox/flox}$ $Apoe^{-/-}$ and $Insr^{flox/\Delta}$ $Apoe^{-/-}$, respectively). **B**, Mean macrophage content in aortic sinus plaques expressed as F4/80-positive area relative to total plaque area in 7 control and 8 haploinsufficient animals at 52 weeks of age. **C** and **D**, The brachiocephalic arteries from 7 control and 8 haploinsufficient animals were dissected free of perivascular fat and arterial lipid was extracted with chloroform/methanol, then analyzed with mass spectrometry. **C**, Abundance of free cholesterol. **D**, Abundance of cholesteryl ester.

Supplemental Figure I



Supplemental Figure II



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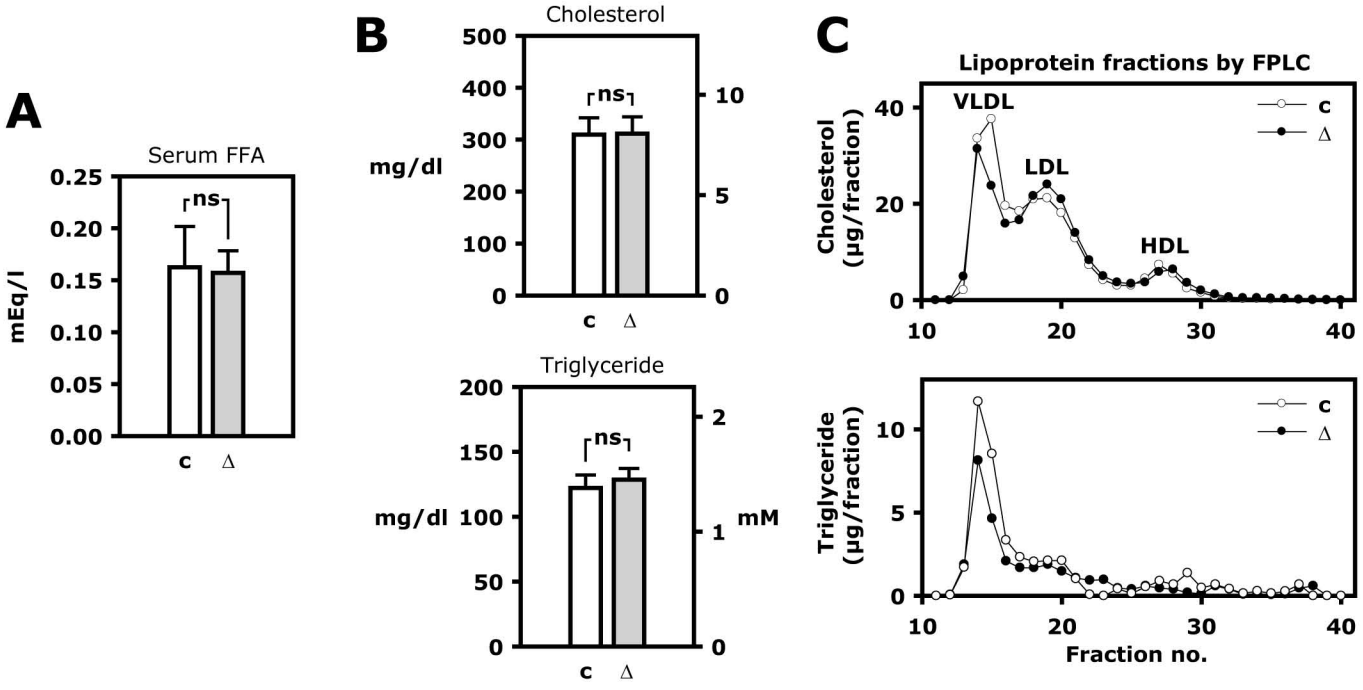
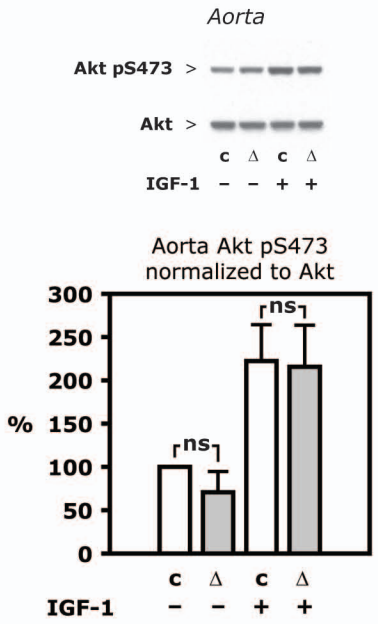


Supplemental Figure IV



Supplemental Figure V