Hyperinsulinemia Does Not Change Atherosclerosis Development in Apolipoprotein E Null Mice

Christian Rask-Madsen, Erica Buonomo, Qian Li, Kyoungmin Park, Allen C. Clermont, Oluwatobi Yerokun, Mark Rekhter, George L. King

Objective—To determine the contribution of hyperinsulinemia to atherosclerosis development.

Methods and Results—Apolipoprotein E (ApoE) null mice that had knockout of a single allele of the insulin receptor (Insr) gene were compared with littermate Apoe null mice with intact insulin receptors. Plasma insulin levels in Insr haploinsufficient/ApoE null mice were 50% higher in the fasting state and up to 69% higher during a glucose tolerance test, but glucose tolerance was not different in the 2 groups. C-peptide levels, insulin sensitivity, and postreceptor insulin signaling in muscle, liver, fat, and aorta were not different between groups, whereas disappearance in plasma of an injected insulin analog was delayed in Insr haploinsufficient/ApoE null mice, indicating that impaired insulin clearance was the primary cause of hyperinsulinemia. No differences were observed in plasma lipids or blood pressure. Despite the hyperinsulinemia, atherosclerotic lesion size was not different between the 2 groups at time points up to 52 weeks of age when measured as en face lesion area in the aorta, cross-sectional plaque area in the aortic sinus, and cholesterol abundance in the brachiocephalic artery.

Conclusion—Hyperinsulinemia, without substantial vascular or whole-body insulin resistance and without changes in plasma lipids or blood pressure, does not change susceptibility to atherosclerosis. (Arterioscler Thromb Vasc Biol. 2012;32:1124-1131.)

Key Words: atherosclerosis • diabetes mellitus • genetically altered mice • insulin resistance • signal transduction

Numerous epidemiological studies have shown that hyperinsulinemia is a major independent risk factor for cardiovascular disease.1-4 An increase in circulating insulin levels is usually considered a surrogate measure of insulin resistance, which is often thought to have a closer causal relationship with cardiovascular disease than hyperinsulinemia.5,6 However, insulin can activate several mechanisms considered proatherosclerotic through direct effects on vascular or inflammatory cells.7-14 Whether hyperinsulinemia changes susceptibility to atherosclerosis independent of associated metabolic changes such as insulin resistance, dyslipidemia, or hypertension is not known. A barrier to answering this question has been the unavailability of an atherosclerosis-susceptible animal model with endogenous hyperinsulinemia but without a substantial impairment of insulin action and with no changes in circulating lipids or blood pressure.

We recently reported that knockout of the insulin receptor targeted to endothelial cells increases atherosclerotic lesion area in the aorta by up to 2.9-fold in the absence of changes in glucose or lipid metabolism.15 Downregulation of endothelial cell vascular cell adhesion molecule-1 by insulin appeared to contribute to this effect.15 Because mice with endothelial cell insulin receptor knockout did not have changes in circulating insulin levels, the results imply that normal levels of insulin protects against atherosclerosis development. However, in that study we could not address the influence, if any, of hyperinsulinemia itself on atherogenesis. Insulin can activate several mechanisms considered proatherosclerotic through direct effects on vascular cells, including increased endothelial cell expression of endothelin-1,15-17 and plasminogen activator inhibitor-1,18,19 and increased smooth muscle cell proliferation.20 Insulin may also augment activation of macrophages infiltrating the vascular wall, and conditional deletion of the insulin receptor (Insr) gene in macrophages has been shown to decrease the rate of atherosclerosis development in some21,22 but not all23 studies.

During characterization of mice bred to create tissue-specific insulin receptor knockout,24 we also created mice with whole-body knockout of a single allele of the insulin receptor combined with total knockout of the apolipoprotein E (ApoE) gene. These animals had hyperinsulinemia but no signs of insulin resistance, suggesting that they would be an attractive model for studying the effect of hyperinsulinemia on atherosclerosis development. Although insulin receptor knockout

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was created by cre recombinase activity in the germline of mice with a “floxed” mutation in the \( \text{Insr} \) gene, animals used for experiments were offspring of cre-negative mice and therefore had an inherited deletion of a single allele of the \( \text{Insr} \) gene. Metabolic studies showed that, compared with littermate controls, these mice had hyperinsulinemia primarily due to decreased insulin clearance, with no detectable impairment of insulin signaling and no change in glucose tolerance or whole-body insulin sensitivity measured by insulin tolerance tests. Plasma lipid levels and blood pressure were not different in the 2 groups. This model has allowed us to determine whether hyperinsulinemia can alter atherosclerosis development.

**Methods**

Additional details of the experimental procedures are included in the online-only Data Supplement.

**Animals**

Cross-breeding of mice with exon 4 of the \( \text{Insr} \) gene flanked by loxP sequences (“floxed”), Tie2-cre transgenic mice, and \( \text{Apoe} \) null mice have been described previously. When breeding \( \text{Apoe} \) null mice homozygous for a floxed \( \text{Insr} \) gene, a whole-body null recombination of a single allele of the \( \text{Insr} \) gene occurred if the female breeder harbored the Tie2-cre transgene, presumably because of cre-mediated recombination in the female germline (Figure I in the online-only Data Supplement), as described previously for this transgene. For the current study, we used breeders negative for the cre transgene, which ensured that a null mutation in a single \( \text{Insr} \) allele was always inherited. Female breeders had a null recombination of a single allele of the \( \text{Insr} \) gene (\( \text{Insr}^{\text{floxed}} \text{Apoe}^{-/-} \)). Male breeders had floxed but intact \( \text{Insr} \) genes (\( \text{Insr}^{\text{floxed}} \text{Apoe}^{+/+} \)). The resultant offspring that were used for experiments had the genotypes \( \text{Insr}^{\text{floxed}} \text{Apoe}^{-/-} \) (“haploinsufficient”) or \( \text{Insr}^{\text{floxed}} \text{Apoe}^{+/+} \) (“controls”) (Figure I in the online-only Data Supplement).

The mice had been partly backcrossed to the C57 background, with previous genetic characterization of animals from the same colony showing that animals had \( 87.6\% \pm 0.8\% \) of the C57BL/6 background, using an array genotyping 377 single-nucleotide polymorphisms. Littermate controls were used in all analysis. Female mice were used for experiments and fed a regular chow diet with \( 9.0\% \) (w/d/wt) fat and \( 0.221 \text{ ppm cholesterol (Mouse Diet 9F, LabDiet). All protocols for animal use and euthanasia were reviewed and approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines.} \)

**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 after insulin stimulation. Anesthesia was induced with pentobarbital. In some animals, insulin or insulin diluent was injected into the vena cava after laparotomy. Blood was drawn by cardiac puncture in a syringe containing EDTA immediately before opening the thorax.

**Glucose and Insulin Tolerance Tests**

Glucose and insulin tolerance tests were performed as described. In \( \text{Apoe} \) null mice with haploinsufficiency of the \( \text{Insr} \) gene (“haploinsufficient mice”), insulin receptor protein expression was reduced in all tissues tested when compared with littermate \( \text{Apoe} \) null controls with a “floxed” but intact \( \text{Insr} \) gene (“control mice”). In liver of haploinsufficient mice, protein expression of the \( \beta \)-subunit of the insulin receptor was 46% of levels in control mouse (Figure 1A and 1C, \( P<0.01 \)) and in skeletal muscle it was 25% of control levels (Figure 2A, \( P<0.01 \)). Similar to what was previously reported for \( \text{Insr}^{+/+} \) mice, body mass was 13% lower in haploinsufficient mice than in controls at 24 weeks of age (Table, \( P=0.02 \)). Dual-energy X-ray absorptiometry scanning showed that fat mass was 12.7% lower and lean mass 4.5% lower in haploinsufficient mice compared with controls (Table), although these differences were not statistically significant. Food intake was 3.3\%±0.6 and 3.4\%±0.6 g/d in control and haploinsufficient mice, respectively (\( P=0.9 \)). Therefore, the lower body mass in the haploinsufficient mice was probably caused primarily by a reduction in fat mass, although more detailed characterization of these changes would require further study.

Intravenous injection of an insulin bolus (50 mU/g body weight) increased insulin receptor expression in phospho-tyrosine immunoprecipitate from liver lysate of control mice, but less so in haploinsufficient mice, in which it was 34±7% of the control value (Figure 1B and 1C, \( P<0.01 \)). Despite reduction in insulin receptor expression and levels of tyrosine-phosphorylated receptors, downstream insulin signaling was not affected, similar to what was previously found in \( \text{Insr}^{+/+} \) mice. In skeletal muscle in the current study, intravenous injection of insulin stimulated phosphorylation at Ser9 of Akt in control and haploinsufficient mice 23.8±4.9-fold and 24.8±1.4-fold, respectively, from the levels of control mice injected with vehicle (Figure 2A and 2B). Similarly, insulin-stimulated phospho-Akt in liver was 245±36\% and 260±49\%, respectively, of the levels of control mice injected with vehicle and in perigonadal fat 305±32\% versus 288±31\% (Figure 2B, all statistically nonsignificant). Insulin-stimulated phosphorylation of glycogen synthase kinase-3\( \beta \) (GSK-3\( \beta \)) at Ser9 also was not assay showed no detectable value in plasma samples obtained before insulin aspart injection.

**Quantification of Atherosclerotic Lesion Size**

Quantification of \textit{en face} atherosclerotic lesion size in the aorta, cross-sectional plaque area in the aortic sinus, and brachiocephalic artery cholesterol abundance was performed as described previously.

**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 the Wilcoxon rank-sum test. All other analyses were made using paired or unpaired \( t \) tests. Statistical significance was defined as \( P<0.05 \). In text and graphs, data are presented as mean±SEM.
organs were isolated and flash frozen. Representative Western blots based on lysate of liver from control (c) and haploinsufficient (Δ) mice (Insr
+/−
ApoE−/−
; Insr
flox/flox
ApoE−/−
, respectively). M indicates molecular weight marker. B, Insulin (50 mU/g) or insulin diluent was injected in the vena cava during pentobarbital anesthesia. After 5 minutes, organs were isolated and flash frozen. Representative Western blots based on phospho-tyrosine immunoprecipitates from liver lysate using tissue from control (c) and haploinsufficient (Δ) mice. C, Average values from 6 control and 5 haploinsufficient animals (left) and from 6 control and 6 haploinsufficient animals (right).

Figure 1. Insulin receptor expression and tyrosine phosphorylation in liver. A, Representative Western blots based on lysate of liver from control (c) and haploinsufficient (Δ) mice (Insr
flox/flox
ApoE−/−
and Insr
flox/−
ApoE−/−
, respectively). M indicates molecular weight marker. B, Insulin (50 mU/g) or insulin diluent was injected in the vena cava during pentobarbital anesthesia. After 5 minutes, organs were isolated and flash frozen. Representative Western blots based on phospho-tyrosine immunoprecipitates from liver lysate using tissue from control (c) and haploinsufficient (Δ) mice. C, Average values from 6 control and 5 haploinsufficient animals (left) and from 6 control and 6 haploinsufficient animals (right).

significantly different in muscle, liver, and fat in the 2 groups (Figure 2A and data not shown). These experiments were repeated with a lower intravenous dose of insulin, 0.5 mU/g, which resulted in plasma insulin concentrations of 214 μU/mL or less in blood sampled just before tissue isolation. Again, insulin-stimulated phospho-Akt and phospho-GSK-3β was similar in the 2 groups in liver, muscle, and fat (Figure II in the online-only Data Supplement).

Hyperinsulinemia without glucose intolerance was previously reported for Insr
+/−
mice.19 To examine whether Insr haploinsufficiency in our mouse model had a similar phenotype, glucose tolerance tests were performed by intraperitoneal injection of glucose in fasting mice. Plasma insulin levels were elevated in haploinsufficient mice at every time point studied (Figure 3A); in the basal condition before glucose injection, it was 50% higher (P<0.05), and 30 minutes after glucose injection it was 69% higher in haploinsufficient mice compared with controls (1.49±0.20 versus 0.88±0.14 ng/mL at 30 minutes, respectively; Figure 3A, P=0.02). Haploinsufficient mice had somewhat better glucose tolerance, although this was not significantly different between the 2 groups (Figure 3B). Furthermore, plasma C-peptide in the random fed state was similar in the 2 groups in the current study, 322±81 pmol in controls and 315±65 pmol in haploinsufficient animals (Table, P=0.9), suggesting that there was no difference in insulin production.

Because hyperinsulinemia was not readily explained by insulin resistance or increased insulin production, we measured insulin clearance. Exogenous insulin was given as an intravenous injection of human insulin with a proline B28 to aspartate mutation (NovoLog, Novo Nordisk), an insulin analog with receptor affinity and potency similar to that of regular human insulin,21 but allowing specific immunodetection of exogenous insulin. Disappearance of B28-Asp insulin in plasma was substantially slower in Insr haploinsufficient mice compared with controls, with plasma concentrations of B28-Asp insulin up to 2.7-fold higher in haploinsufficient animals (Figure 3C, P<0.01 for area under the curve).

During an insulin tolerance test in the random fed state, mice with Insr haploinsufficiency showed a decrease in blood glucose no different than in control mice during a course of 2 hours, indicative of similar insulin sensitivity (Figure 3D, P>0.3). Overall, this characterization is compatible with hyperinsulinemia caused primarily by decreased insulin clearance.

ApoE null mice spontaneously develop hypercholesterolemia on a regular chow diet. In haploinsufficient animals, serum concentrations of free fatty acids (Figure IIIA in the online-only Data Supplement) and plasma concentrations of total cholesterol and total triglyceride (Figure IIIB in the online-only Data Supplement) were not different in haploinsufficient and control mice. Distribution of cholesterol and triglycerides in different lipoprotein moieties was measured as lipid concentration in fractions separated by fast protein liquid chromatography and was not different in the 2 groups (Figure IIIC in the online-only Data Supplement). Therefore, Insr haploinsufficiency in an ApoE null background does not change major lipid parameters involved in atherosclerosis development.

Analysis of protein expression in the aorta showed that the β-subunit of the insulin receptor was reduced by 50.0±8.4% (Figure 4A and data not shown, P<0.001). Expression of eNOS protein in the aorta was not different in the 2 groups (Figure 4A, P=0.96). Vascular cell adhesion molecule-1, which is upregulated in endothelial cells in mice with insulin receptor knockout targeted to endothelial cells,15 or endothelin-1 were also not different between haploinsufficient and control animals (Figure 4A). Vascular wall insulin sensitivity was assessed by insulin-stimulated Akt phosphorylation in vivo. Akt Ser473 phosphorylation in the aorta after an intravenous insulin bolus, as in muscle, liver, and fat, was similar between groups (Figure 4B). The insulin-stimulated level relative to control animals injected with vehicle was 397±93% in controls and 351±45% in haploinsufficient animals, respectively (Figure 4B, P=0.6). Decreased insulin receptor expression could potentially change IGF-1 signaling by decreasing the number of hybrid insulin/IGF-1 receptors or by other
mechanisms. However, intravenous injection of IGF-1 increased phosphorylation of Akt in the aorta to the same degree in control and haploinsufficient mice (to 222±42% and 216±48% of control levels, Figure IV in the online-only Data Supplement, *P* < 0.9). There was no difference between the 2 groups of animals in terms of systolic or diastolic blood pressure or pulse rate (Table).

Atherosclerosis in the aorta was measured as the fraction of the surface area in *en face* flat preparations stained by Sudan IV (Figure 5A through 5D). At 24 weeks of age, the lesion area, on average, was almost identical in the 2 groups; it was 1.5±0.3% in haploinsufficient animals and 1.6±0.3% in controls (Figure 5B, *P* = 0.8). At 52 weeks, lesion area remained without significant differences (16.9±3.5% and 14.0±2.5% in control and haploinsufficient mice, respectively, Figure 5D, *P* = 0.6). At this age, the cross-sectional plaque area in the aortic sinus and the macrophage content in the aortic sinus were also not statistically different in the 2 groups (plaque area, 541 489±632 632 μm² and 379 405±124 414 μm², respectively; Figure 5E and 5F, *P* = 0.2; F4/80-positive area relative to plaque area, 14.1±2.9% and 12.4±2.4%, respectively; Figure VA and B in the online-only Data Supplement, *P* = 0.6).

As a separate analysis of atherosclerotic plaque development, cholesterol content was analyzed in extracts of the brachiocephalic artery at 36 weeks of age. This measure has been shown to correlate well with lesion area in histological cross sections. Free cholesterol abundance was 8.4±2.4 and 8.6±1.6 nmol (Figure VC in the online-only Data Supplement) and cholesterol ester was 8.6±3.6 and 8.4±1.9 nmol (Figure VD in the online-only Data Supplement) in haploinsufficient animals and littermate controls, respectively (*P* > 0.9). This indicated that no significant difference existed in atherosclerotic lesion size in

### Table. Characteristics of Haploinsufficient Animals (Δ) and Controls

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Unit</th>
<th>Controls Mean±SEM</th>
<th>Δ Mean±SEM</th>
<th>n Controls; Δ</th>
<th><em>P</em></th>
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<tr>
<td>Body mass</td>
<td>g</td>
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<tr>
<td>Lean mass</td>
<td>g</td>
<td>6.4±1.9</td>
<td>5.6±1.5</td>
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<tr>
<td>Fat mass</td>
<td>g</td>
<td>21.9±1.0</td>
<td>20.9±0.9</td>
<td>12; 7</td>
<td>0.4</td>
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<tr>
<td>Food intake</td>
<td>g/d</td>
<td>3.3±0.6</td>
<td>3.4±0.6</td>
<td>7; 8</td>
<td>0.9</td>
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<tr>
<td>C-peptide</td>
<td>pmol</td>
<td>322±81</td>
<td>315±65</td>
<td>9; 10</td>
<td>0.9</td>
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<tr>
<td>Fasting plasma cholesterol</td>
<td>mg/dL</td>
<td>295±31</td>
<td>313±23</td>
<td>10; 10</td>
<td>0.7</td>
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<tr>
<td>Fasting plasma triglyceride</td>
<td>mg/dL</td>
<td>131±30</td>
<td>106±7</td>
<td>8; 9</td>
<td>0.4</td>
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<td>Systolic blood pressure</td>
<td>mm Hg</td>
<td>113±3</td>
<td>114±3</td>
<td>11; 11</td>
<td>0.6</td>
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<tr>
<td>Diastolic blood pressure</td>
<td>mm Hg</td>
<td>92±3</td>
<td>95±3</td>
<td>11; 11</td>
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<td>Pulse</td>
<td>1/min</td>
<td>522±32</td>
<td>549±24</td>
<td>11; 11</td>
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haploinsufficient mice and their controls when measured at different time points, in various locations, and by different approaches.

Discussion

High circulating insulin concentrations present in conditions such as obesity and type 2 diabetes have for decades been suspected to promote atherosclerosis through direct effects on the vascular wall. However, animal models to test this hypothesis have been lacking. Hyperinsulinemia in models of obesity and diabetes is associated with changes in plasma lipids which confound the association between plasma insulin and atherosclerosis development. Furthermore, hyperinsulinemia is usually associated with significant impairment of insulin signaling in the vascular wall, which in itself can promote atherosclerosis. In the current study, we describe a mouse model with levels of hyperinsulinemia that are relevant for human disease and primarily due to decreased insulin clearance, with little impairment of insulin action in the vasculature or in other tissues. Atherosclerosis development in these mice and their controls is caused by hypercholesterolemia due to deletion of the Apoe gene. However, we found no difference in atherosclerotic lesion size in hyperinsulinemic mice compared with controls. These data indicate that abnormally high insulin concentrations alone, without substantial impairment of vascular insulin signaling, and in the absence of significant changes in glucose or lipid metabolism, are not sufficient to accelerate atherosclerosis.

Plasma insulin in the Insr haploinsufficient mice was 50% higher than in controls in the fasting state and up to 69% higher during a glucose tolerance test. This level of hyperinsulinemia is appropriate as a model of hyperinsulinemia in humans at risk for cardiovascular disease. For example, in the Quebec Heart Study, the mean plasma insulin in the case group of patients with cardiovascular disease was 18% higher than in control subjects (92.1 versus 78.2 pmol), with an odds ratio of 1.7 for each standard deviation of increase in plasma insulin. In human conditions and in animal models of obesity and insulin resistance, hyperinsulinemia is often an index of peripheral insulin resistance. Therefore, it may seem counterintuitive that hyperinsulinemia in the haploinsufficient mice in the current study is present together with preserved insulin sensitivity and glucose tolerance. However, it is possible that clearance of insulin, which involves binding of the hormone to its receptor on hepatocytes, may be affected more...
by a reduction in insulin receptor numbers than postreceptor insulin signaling or insulin-stimulated glucose uptake.

In the current study, neither mice used for experiments nor their parents carried a cre transgene. Therefore, haploinsufficiency of the Insr gene in Insr flox/ flox Apoe −/− mice was due to an inherited mutation and cre-mediated deletion in any tissue would not have occurred. The metabolic phenotype of Insr flox/ flox Apoe −/− mice may be modified by the Apoe null mutation but is generally compatible with data from Insr +/+ mice, in which the Insr deletion was created by targeted mutation in stem cell culture. In the publication first describing the phenotype of Insr +/+ mice, it was noted that animals had hyperinsulinemia in the fasting state and during glucose tolerance test but no glucose intolerance. Subsequent studies of these mice showed a modest reduction or no reduction in insulin-signaling events in liver, muscle, or fat, such as p85 coimmunoprecipitation with phospho-tyrosine or IRS proteins or PI3K activity in phospho-tyrosine immunoprecipitates.

In the current study, we observed no decrease in insulin signaling between haploinsufficient and control mice over a 100-fold range in insulin dose, from 0.5 to 50 mU/g. In contrast, mice fed a high-fat diet develop insulin resistance with considerable inhibition of insulin-stimulated phospho-Akt at insulin doses similar to the high dose (50 mU/g) used in the current study. Haploinsufficient mice had a modest reduction of body mass relative to control mice, although this had no apparent effect on insulin sensitivity, other metabolic parameters, or circulating lipids. Insulin sensitivity in both haploinsufficient and control mice in our study was probably affected in adipose tissue,31–33 It is possible that haploinsufficient mice could have decreased insulin signaling compared with control mice at an even lower insulin dose or that insulin sensitivity, which was not different between the 2 groups in insulin tolerance tests, would be impaired during a euglycemic hyperinsulinemic clamp. Regardless, the primary cause of hyperinsulinemia in haploinsufficient mice appears to be impaired insulin clearance. Measurement of insulin clearance in Insr +/+ mice has not been published, but mice with deletion of the Insr gene specifically in hepatocytes have plasma insulin concentrations that are >20-fold higher than their controls,34 confirming that the majority of insulin clearance occurs in the liver after binding of the hormone to its receptor.8,35

Plasma cholesterol and triglyceride in plasma and in lipoprotein fractions were not different in haploinsufficient animals and controls. This made it possible to compare the effect of hyperinsulinemia on atherosclerosis when accompanied with no or only mild insulin resistance, without any confounding effect of changes in plasma lipids or blood pressure. Insulin receptor knockout mice with a transthyretin transgene, which rescues these mice from neonatal death by expression of <10% of control levels of insulin receptor protein in the liver, have decreased atherosclerosis.36 However, these animals have changes in several determinants of atherosclerosis development, including abolished insulin signaling.
in the aorta and reduced plasma VLDL and LDL cholesterol as well as reduced hepatic VLDL secretion.36

In mice with normal circulating insulin levels and loss of insulin signaling in endothelial cells, atherosclerotic lesion area was increased in the aorta by up to 2.9-fold.15 Together with data from the current study, these results suggest that in the metabolic syndrome and type 2 diabetes, activation of potentially proatherosclerotic mechanisms in vascular cells by hyperinsulinemia has little effect on atherogenesis, whereas substantial insulin resistance in endothelial cells by itself can promote atherosclerosis development. This implies that in terms of the contribution of abnormal vascular insulin signaling to atherosclerosis, our efforts may be better focused on improving endothelial cell insulin resistance than preventing effects of hyperinsulinemia on the vascular wall.

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Figure 5. Atherosclerosis. A through D, Aortas were dissected free of perivascular tissue, stained with Sudan IV, and mounted in a flat preparation (en face preparation). A and C, Representative photographs of aortas from an haploinsufficient mouse and its littermate control at 24 weeks (A) and 52 (C) weeks of age. B and D, Average atherosclerotic lesion area in 10 control (c) and 10 haploinsufficient (Δ) mice (Insr flox/Apo e -/- and Insr flox/Apo e -/- , respectively) at 24 weeks of age (B) and in 8 control and 10 haploinsufficient animals at 52 weeks of age (D). E and F, Cryosections of aortic sinus were made from animals at 52 weeks of age and stained with hematoxylin and eosin. E, Representative microphotographs; scale bar represents 500 μm. F, Mean plaque area in 7 control and 8 haploinsufficient animals.

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Disclosures
Dr Rekhter is an employee of Eli Lilly & Co.

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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. 1A). Littermate controls were used in all analysis comparing Insr^{flox/flox} Apoe^{−/−} (“controls”) and Insr^{flo/x/Δ} 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), Na3VO4 (1 mM), leupeptin (10 µg/ml), apro tinin (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


Supplementary figure legends

**Supplementary figure I. Genetic modifications and mouse breeding.**
Diagram showing generation of $\text{Insr}^{\text{flox}/\Delta}$ $\text{Apoe}^{-/-}$ and littermate controls ($\text{Insr}^{\text{flox}/\text{flax}}$ $\text{Apoe}^{-/-}$) 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 ($\text{Insr}$) 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 ($\Delta$) mice ($\text{Insr}^{\text{flox}/\text{flax}}$ $\text{Apoe}^{-/-}$ and $\text{Insr}^{\text{flox}/\Delta}$ $\text{Apoe}^{-/-}$, 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 ($\Delta$) mice ($\text{Insr}^{\text{flox}/\text{flax}}$ $\text{Apoe}^{-/-}$ and $\text{Insr}^{\text{flox}/\Delta}$ $\text{Apoe}^{-/-}$, 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 ($\Delta$) mice, $\text{Insr}^{\text{flox}/\text{flax}}$ $\text{Apoe}^{-/-}$ and $\text{Insr}^{\text{flox}/\Delta}$ $\text{Apoe}^{-/-}$, 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 ($\Delta$) mice ($\text{Insr}^{\text{flox}/\text{flax}}$ $\text{Apoe}^{-/-}$ and $\text{Insr}^{\text{flox}/\Delta}$ $\text{Apoe}^{-/-}$, 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 II

A

Muscle

Liver

Fat

Insulin 0.5 mU/g:

Insulin: - - + +

B

Muscle Akt pS473

Liver Akt pS473

Fat Akt pS473

Supplemental Figure III

A

Serum FFA

Cholesterol

Triglyceride

B

C

Lipoprotein fractions by FPLC

VLDL

LDL

HDL

Fraction no.
Supplemental Figure IV

Aorta

Akt pS473
Akt
IGF-1

Aorta Akt pS473 normalized to Akt

Supplemental Figure V

A

F4/80
DAPI

B

Plaque macrophages

C

Brachiocephalic a.
free cholesterol

D

Brachiocephalic a.
cholesteryl ester