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Leptin Receptor-induced STAT3-Independent Signaling Pathways are Protective against Atherosclerosis in a Murine Model of Obesity and Hyperlipidemia

Wei Luo, Peter F. Bodary, Yuechun Shen, Kevin J. Wickenheiser, Miina K. Öhman, Chiao Guo, Kristina L. Bahrou, Martin G. Myers Jr., and Daniel T. Eitzman

University of Michigan, Department of Internal Medicine, Cardiovascular Research Center, Ann Arbor, Michigan, USA

Abstract

AIMS—Leptin is an adipocyte-derived hormone that has been shown to exert both beneficial metabolic effects and potentially adverse vascular effects in preclinical studies. The primary aim of this study was to determine the effects of leptin receptor signaling pathways on atherosclerosis in the setting of obesity and hyperlipidemia.

METHODS AND RESULTS—Mice were generated with deficiency of apolipoprotein E (*ApoE*^{−/−}) and either wild-type leptin receptor expression (*Lepr*^{+/+}, *ApoE*^{−/−}), mutant leptin receptor expression defective in all leptin receptor signaling pathways (*Lepr*^{db/db}, *ApoE*^{−/−}), or mutant leptin receptor expression with selective deficiency of leptin receptor-STAT3 signaling (*Lepr*^{s/s}, *ApoE*^{−/−}). At 27 weeks of age (including 7 weeks on a western chow diet), *Lepr*^{db/db}, *ApoE*^{−/−} developed severe obesity, hypercholesterolemia, and increased atherosclerosis compared to *Lepr*^{+/+}, *ApoE*^{−/−} mice. Despite similar obesity and hyperlipidemia to *Lepr*^{db/db}, *ApoE*^{−/−} mice, *Lepr*^{s/s}, *ApoE*^{−/−} developed less atherosclerosis than *Lepr*^{db/db}, *ApoE*^{−/−} mice. Adipose tissue macrophage content, monocyte chemoattractant protein-1 and fatty-acid-binding protein 4 levels were also reduced in *Lepr*^{s/s}, *ApoE*^{−/−} mice compared to *Lepr*^{db/db}, *ApoE*^{−/−} mice.

CONCLUSIONS—In a mouse model of obesity and hyperlipidemia, leptin receptor-mediated STAT3-independent signaling pathways confer protection against atherosclerosis. These differences occur independently of leptin effects on energy balance.

Keywords

obesity; leptin; hyperlipidemia; atherosclerosis; murine model

Introduction

Leptin is an adipocyte-derived hormone that plays a critical role in the regulation of appetite and metabolism¹. In addition, leptin receptor signaling pathways may play a role in many other physiologic and pathologic processes since multiple cell types express the leptin receptor². Leptin levels rise in proportion to fat mass but resistance to the central effects of

Corresponding author: Daniel T. Eitzman, MD, University of Michigan, Cardiology, 7301A MSRB III, 1150 West Medical Center Drive, Ann Arbor, MI 48109-0644, USA, Tel: +1-734-647-9865, Fax: +1-734-936-2641, deitzman@umich.edu.

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leptin occur, which attenuate the appetite suppressive effects of leptin. Sensitivity to the effects of leptin may also vary depending on cell type^{3, 4}.

Although leptin has been linked to atherosclerosis in some preclinical studies⁵, the specific role of leptin on atherosclerosis is difficult to determine since the state of complete leptin or leptin receptor deficiency leads to extreme obesity and hyperlipidemia, compared to lean control mice, which may overwhelm other direct vascular effects of leptin^{6, 7}.

Mice lacking STAT-3 (signal transducer and activator of transcription 3)-dependent leptin receptor signaling (*Lepr^{s/s}*) develop obesity similarly to mice with complete abrogation of leptin receptor signaling (*Lepr^{db/db}*)^{8, 9}. These mice thus represent a useful model to test the effects of STAT3-independent leptin receptor signaling pathways in the setting of obesity.

In the present study, we determined the role of STAT3-independent leptin receptor signaling pathways on atherosclerosis in the setting of severe obesity and hyperlipidemia.

Material and Methods

Mice

ApoE^{-/-} mice and heterozygous *Lepr^{db/+}* mice on the C57BL6/J background were purchased from the Jackson Laboratory (Bar Harbor, ME) and genotyped as previously described⁵. *Lepr^{s/+}* mice have been generated as previously described⁹ and have been backcrossed to the C57BL6/J strain background for >10 generations. Mice were fed a standard laboratory rodent diet (#5001, TestDiet, Richmond, IN) in specific pathogen-free facilities ad libitum for 20 weeks of age followed by a Western diet (TD88137, Harlan, WI) for an additional 7 weeks. Both male and female mice were used in this study and no significant gender differences were observed. The procedures conform to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and were approved by the University of Michigan Committee on Use and Care of Animals.

Dual-energy X-ray absorptiometry (DEXA)

Measurements of body fatness were made by dual-energy X-ray absorptiometry (DEXA) using a Lunar PixiMus densitometer (GE, software version 1.4x; Madison, WI). Mice were anesthetized with intraperitoneal pentobarbital (100mg/kg) and analyzed as previously described¹¹.

Bone Marrow Transplantation

At 10 weeks of age, *ApoE^{-/-}* mice were irradiated and bone marrow transplantation (BMT) was performed as previously described¹². Mice were given a Western diet (TD88137, Harlan, WI) beginning 5 weeks following the BMT for 7 weeks, after which mice were euthanized.

Atherosclerosis Quantification and Characterization

For quantification of atherosclerosis, animals were euthanized under intraperitoneal pentobarbital anesthesia (100mg/kg) and arterial trees were dissected and stained with Oil Red O. The surface lesion area was analyzed with Image-Pro Plus software (Media Cybernetics, Bethesda, MD) and expressed as a percentage of total surface area examined as previously reported⁵.

For analysis of lesion thickness, cross-sections from paraffin-embedded blocks were analyzed at the level of the aortic root. 4 cross sections were analyzed from each mouse. To

quantitate lesion macrophages and vascular smooth muscle cells, immunohistochemistry with antibodies to Mac3 (1:100 dilution; BD Biosciences, San Jose, CA) and α -smooth muscle cell actin (1:1000 dilution, Chemicon, Billerica, MA) were used as previously described¹¹ and the lesion area occupied by positively stained cells was expressed as a percentage of total atherosclerotic lesion area. The area of lesion occupied by collagen was determined after staining with Masson trichrome (Sigma, St Louis, MO) (identified as blue staining component of plaque). The area of lesion occupied by lipid clefts (clear, nonstaining vacuoles) was quantitated after hematoxylin and eosin staining. Collagen and lipid clefts were also expressed as a percentage of total lesion area.

Plasma Measurements

Plasma samples were collected by ventricular puncture at time of euthanasia after 5-hour fasting. Leptin, Mcp-1 and FABP4 were measured with commercially available murine ELISA kits (Leptin - Crystal Chemical Inc, Downers Grove, IL) (Mcp-1 – R & D Systems, Minneapolis, MN) (FABP4- Biovender, Candler, NC) according to manufacturers' instructions. Glucose (Sigma, St. Louis, MO), insulin (Crystal Chemical Inc, Downers Grove, IL), and total cholesterol concentrations (Wako, Richmond, VA) were measured with colorimetric assays.

Adipose tissue inflammation

For analysis of adipose tissue macrophage content, paraffin sections of visceral fat were identified with a rat anti-mouse Mac-3 monoclonal antibody (1:100 dilution; BD Biosciences, San Jose, CA) and analyzed as previously reported¹¹.

Statistical Analysis

All parameters were evaluated by 1-way ANOVA and Tukey's Studentized Range (HSD) test or Student's t-test when comparisons were made between 2 groups. Values of $P < 0.05$ were considered significant and expressed as mean $\pm$ SEM.

Results

Effect of leptin-receptor mutations on metabolic parameters in *ApoE*^{-/-} deficient mice

Body weight was markedly elevated in *Lepr*^{db/db}, *ApoE*^{-/-} and *Lepr*^{s/s}, *ApoE*^{-/-} mice compared to *Lepr*^{+/+}, *ApoE*^{-/-} mice (Table 1). *Lepr*^{db/db}, *ApoE*^{-/-} and *Lepr*^{s/s}, *ApoE*^{-/-} mice also developed similar hyperleptinemia and hypercholesterolemia that were both higher than *Lepr*^{+/+}, *ApoE*^{-/-} mice (Table 1). Levels of fasting insulin were higher in obese *Lepr*^{s/s}, *ApoE*^{-/-} mice and *Lepr*^{db/db}, *ApoE*^{-/-} mice but glucose levels were not significantly different between groups (Table 1).

Effect of mutant leptin receptor signaling on total body fat mass and atherosclerosis

Total body fat mass was similar between the *Lepr*^{db/db}, *ApoE*^{-/-} and *Lepr*^{s/s}, *ApoE*^{-/-} mice and both of these groups had significantly greater fat mass than the *Lepr*^{+/+}, *ApoE*^{-/-} mice (Figure 1A–D). Despite a similar level of obesity and hyperlipidemia to *Lepr*^{db/db}, *ApoE*^{-/-} mice, the atherosclerosis in *Lepr*^{s/s}, *ApoE*^{-/-} was less than in *Lepr*^{db/db}, *ApoE*^{-/-} mice and not significantly greater than in *Lepr*^{+/+}, *ApoE*^{-/-} mice (Figure 1E–H). Aortic root mean cross-sectional atherosclerotic lesion area was also greater in *Lepr*^{db/db}, *ApoE*^{-/-} mice (0.12 ± 0.02 mm²) compared to *Lepr*^{s/s}, *ApoE*^{-/-} mice (0.07 ± 0.01 mm², $p < 0.04$) and *Lepr*^{+/+}, *ApoE*^{-/-} mice (0.04 ± 0.02 mm², $p < 0.04$). This greater lesion area in the *Lepr*^{db/db}, *ApoE*^{-/-} mice was associated with increased lesion area occupied by lipid clefts compared to *Lepr*^{s/s}, *ApoE*^{-/-} and *Lepr*^{+/+}, *ApoE*^{-/-} mice (Figure 2A–D), and reduced collagen staining (Figure 2E–H). Lesion area occupied by macrophages ($4.5 \pm 3.15\%$ in *Lepr*^{db/db}, *ApoE*^{-/-}

mice, $2.14 \pm 0.72\%$ in *Lepr^{s/s}, ApoE^{-/-}* and $6.37 \pm 2.25\%$ in *Lepr^{+/+}, ApoE^{-/-}* mice), and smooth muscle cells ($5.65 \pm 0.93\%$ in *Lepr^{db/db}, ApoE^{-/-}* mice, $7.36\% \pm 3.07\%$ in *Lepr^{s/s}, ApoE^{-/-}* and $5.04 \pm 1.29\%$ in *Lepr^{+/+}, ApoE^{-/-}* mice) were not different between the groups of mice.

Effect of leptin receptor mutations on adipose tissue inflammation, Mcp-1 and FABP4 levels

Since obesity-related effects on atherosclerosis may be related to inflammatory changes in adipose tissue¹¹, we analyzed the macrophage content of epididymal fat depots from leptin receptor mutant and wild-type mice. Visceral adipose tissue from *Lepr^{db/db}, ApoE^{-/-}* mice contained a greater proportion of macrophages compared to *Lepr^{s/s}, ApoE^{-/-}* and *Lepr^{+/+}, ApoE^{-/-}* mice (Figures 3A – 3D). Plasma levels of Mcp-1 and FABP4 were both higher in the plasma of *Lepr^{db/db}, ApoE^{-/-}* mice compared to *Lepr^{s/s}, ApoE^{-/-}* mice (Figures 3E – 3F).

Effect of mutant leptin receptor bone marrow transplantation on atherosclerosis and metabolic parameters in *ApoE^{-/-}* mice

To examine whether the increased atherosclerosis noted in the *Lepr^{db/db}, ApoE^{-/-}* mice was attributable to the lack of a signaling leptin receptor on circulating blood elements, bone marrow transplantation was performed from *Lepr^{db/db}, ApoE^{-/-}* to *Lepr^{+/+}, ApoE^{-/-}* (n=10), *Lepr^{s/s}, ApoE^{-/-}* to *Lepr^{+/+}, ApoE^{-/-}* (n=10), and *Lepr^{+/+}, ApoE^{-/-}* to *Lepr^{+/+}, ApoE^{-/-}* mice (n=10). The bone marrow derived leptin receptor status did not affect Oil Red O stained atherosclerotic lesion area (3.34 ± 0.49 , 4.07 ± 0.36 and $3.73 \pm 0.35\%$, respectively, p=NS). There were no significant differences in body weight (23.0 ± 1.6 , 23.8 ± 1.5 and 24.0 ± 2.7 grams respectively, p=NS) or fat mass related to the leptin receptor status of the donor marrow (19.9 ± 1.6 , 21.6 ± 2.6 and $20.7 \pm 1.3\%$ respectively, p=NS). Plasma levels of Mcp-1 were also not different between the groups (71.7 ± 6.2 , 78.4 ± 8.8 and 75.0 ± 9.2 pg/ml respectively, p=NS).

Discussion

Obesity is associated with an increased risk of cardiovascular disease and all cause mortality¹³. Although several molecular links between obesity and vascular disease have been proposed, the specific factors responsible for the elevated vascular risk remain unclear¹⁴. Leptin, a hormone produced by adipocytes, has effects on multiple cell types including neurons, hepatocytes, and cells involved in atherosclerosis^{15–18}. Pre-clinical animal studies have shown both positive and negative effects of leptin receptor signaling on processes implicated in vascular disease^{5, 19, 20}.

The vast majority of obese subjects have elevated leptin levels and these subjects do not respond well to the appetite suppressive effects of leptin²¹. This is at least partly due to central leptin resistance²¹. Whether other peripheral leptin receptor cellular pools remain sensitive or become resistant to the effect of leptin in obese states remains unclear, although differential sensitivity to the effects of leptin on physiologic vascular endpoints has been demonstrated⁴. Thus, in the setting of obesity, the effects of leptin are complex and may reflect heterogeneous leptin receptor resistance to chronically elevated circulating leptin levels.

Leptin has been shown to affect vascular phenotypes in mice^{5, 6, 12, 19, 22}, however the specific role of leptin in vascular disease remains unclear because of the confounding metabolic consequences of leptin deficiency⁶. Animal models in which leptin receptor signaling pathways are targeted in the setting of severe obesity, with elevated endogenous

leptin levels, may be more relevant to the human condition and provide unique insight towards the effects of leptin in vascular disease.

Similar to other cytokine receptors, the leptin receptor signals by activating a Jak family tyrosine kinase (Jak2) that phosphorylates tyrosine residues (including Tyr985, Tyr1077 and Tyr1138) of the leptin receptor during leptin stimulation². Leptin can therefore signal through activation of several different receptor-mediated pathways. The relevance of these pathways toward energy balance has been established by the analysis of mice in which the leptin receptor gene has been substituted by a mutant leptin receptor containing a Tyr1138-Ser substitution that is defective in STAT3 binding⁹. These mice develop obesity similar to that observed in *Lepr^{db/db}* mice, although other phenotypes associated with leptin receptor deficiency are less affected, indicating the relevance of other leptin receptor-mediated signaling pathways⁸. Since leptin may be useful as therapy for some patient populations²³ and could become useful as a weight loss treatment to a larger population as mechanisms of leptin resistance are uncovered²⁴, it is critical to study the effects of leptin receptor signaling on atherosclerosis in obese animal models.

The results of the current studies were surprising as mice with partially intact leptin receptor signaling were markedly protected from atherosclerosis compared to mice completely deficient in leptin receptor signaling. Although the lesion contributions of smooth muscle cells and macrophages were similar between the obese groups of mice, evidence of reduced lipid and increased collagen was apparent in the *Lepr^{s/s}, ApoE^{-/-}* mice, suggesting that atherosclerotic plaques from these mice may be more stable compared to plaques from *Lepr^{db/db}, ApoE^{-/-}* mice. These results are not confounded by differences in obesity as weight was similar between the 2 groups of mice with leptin receptor mutations. Cholesterol levels were also similar between the groups, although we cannot rule out an effect of other unmeasured lipid fractions, difference in lipid particle composition, or differences in lipid oxidation status as these lipid characteristics were not measured in the current study. Accelerated atherosclerosis in the setting of obesity may be due to associated diabetes however no differences in fasting glucose were observed between *Lepr^{db/db}, ApoE^{-/-}* mice and *Lepr^{s/s}, ApoE^{-/-}* mice in this study.

Other factors associated with obesity may therefore be more important than diabetes in atherogenesis such as the low grade inflammatory state that is associated with excessive visceral adiposity²⁵. This systemic inflammatory state may be initially triggered by local infiltration of macrophages into obese adipose depots²⁵. *Lepr^{s/s}, ApoE^{-/-}* mice showed reduced macrophage content in epididymal fat pads compared to *Lepr^{db/db}, ApoE^{-/-}* mice which may have played a role in the protection from atherosclerosis, although a causal relationship has not been proven. Future adipose tissue transplantation experiments may be useful to resolve this issue. We have also previously shown that elevated systemic levels of Mcp-1 are an excellent marker of inflammatory visceral adipose tissue and that endothelial Mcp-1 plays a causal role in atherosclerosis induced by inflammatory adipose tissue²⁶. Consistently, Mcp-1 levels in *Lepr^{db/db}, ApoE^{-/-}* mice were higher compared to *Lepr^{s/s}, ApoE^{-/-}* mice. The protective effects of leptin receptor signaling were not due to bone marrow-derived leptin receptor cellular pools, based on bone marrow transplant experiments, although we cannot rule out an effect of bone marrow-derived leptin receptor signaling on atherosclerosis in the setting of severe obesity.

To further explore possible mediators of a heightened inflammation state in obese mice we measured FABP4. FABP4 deficiency states are associated with both reduced fat inflammation and atherosclerosis^{27, 28}. In addition, FABP4 has been shown to induce Mcp-1 expression²⁷. FABP4 levels were elevated in *Lepr^{db/db}, ApoE^{-/-}* mice compared to

Lepr^{s/s}, *ApoE^{-/-}* mice. Varying degrees of hepatic steatosis or inflammation may also influence atherosclerosis, although this variable was not studied in the present work.

Although we cannot establish a causal role of the mediators downstream of leptin receptor signaling responsible for differences in adipose inflammation and atherosclerosis, it is clear that in the setting of obesity and hyperlipidemia, *Lepr*-STAT3-independent signaling is providing protection against atherosclerosis that is associated with reduced adipose tissue inflammation and circulating inflammatory mediators. Identification of the downstream mediators for this effect is critical as these pathways may provide targets for modulating the adverse vascular consequences of obesity.

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Figure 1. Effect of leptin receptor signaling pathways on fat mass and atherosclerosis
 Representative DEXA images of mice. A) *Lepr*^{+/+}, *ApoE*^{-/-} B) *Lepr*^{sl/sl}, *ApoE*^{-/-} C)
Lepr^{db/db}, *ApoE*^{-/-}. D) % of body fat. Representative en face view of aortic tree stained with
 Oil Red O E) *Lepr*^{+/+}, *ApoE*^{-/-}. F) *Lepr*^{sl/sl}, *ApoE*^{-/-}. G) *Lepr*^{db/db}, *ApoE*^{-/-}. H) % lesion
 surface area. N=10–12 per group. **P*<0.05.

Figure 2. Effect of leptin receptor signaling pathways on components of atherosclerosis
H&E staining of aortic root. A) *Lepr*^{+/+}, *ApoE*^{-/-}. B) *Lepr*^{s/s}, *ApoE*^{-/-}. C) *Lepr*^{db/db}, *ApoE*^{-/-}. D) % Lipid clefts area. Masson trichrome staining of aortic root to show collagen as blue. E) *Lepr*^{+/+}, *ApoE*^{-/-}. F) *Lepr*^{s/s}, *ApoE*^{-/-}. G) *Lepr*^{db/db}, *ApoE*^{-/-}. H) % collagen area. N=6 per group. **P*<0.05. Magnification is 10×.

Figure 3. Effect of leptin receptor signaling pathway on macrophage content of epididymal fat tissue (immunohistochemical staining with macrophage specific Mac-3 antibody)

A) *Lepr*^{+/+}, *ApoE*^{-/-}. B) *Lepr*^{s/s}, *ApoE*^{-/-}. C) *Lepr*^{db/db}, *ApoE*^{-/-}. D) % Mac-3 stained cells. E) Plasma MCP-1 level. F) Plasma FABP4 level. N=6–8 per group. **P*<0.05. Arrows point to Mac-3 positive stained cells (brown). Magnification is 40×, arrows showing stained cells.

Table 1

Effect of leptin receptor signaling pathways on metabolic parameters.

	Lepr^{+/+}, ApoE^{-/-}	Lepr^{s/s}, ApoE^{-/-}	Lepr^{db/db}, ApoE^{-/-}
Body weight (gram)	28.12±2.47	58.61±2.03 *	63.16±3.52 *
Leptin (ng/ml)	24.04±12.53	99.62±14.4 *	127.79±34.36 *
Cholesterol E (mg/dl)	998.8±83.23	2202.1±227.24 *	1948.1±244.71 *
Insulin (ng/ml)	0.72±0.14	7.63±2.36 *	3.7±0.73 *
Glucose (mg/dl)	204.44±17.71	165.79±9.3	223.54±43.79

Lepr^{+/+}, ApoE^{-/-}; *Lepr^{s/s}, ApoE^{-/-}*; and *Lepr^{db/db}, ApoE^{-/-}* mice. N=6–8 per group.

* indicate $P < 0.05$ compared to *Lepr^{+/+}, ApoE^{-/-}*.