

Effect of Leptin on Arterial Thrombosis Following Vascular Injury in Mice

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OBESITY IS AN INDEPENDENT risk factor for the complications of atherosclerotic vascular disease, such as myocardial infarction and stroke.¹ Since these complications are due to acute thrombosis,² the development of obesity may elicit a prothrombotic state. Alterations in coagulation and fibrinolytic factors with the development of obesity have been described,³⁻⁵ but causal relationships with these factors and the development of thrombosis have not been established in obesity.

The adipocyte-derived hormone leptin has a stimulatory effect on platelet aggregation induced by adenosine diphosphate.⁶ Since leptin levels increase with the development of obesity, leptin may play a role in the thrombotic vascular complications associated with obesity. To characterize the *in vivo* role of leptin on the development of vascular thrombosis, we studied mice deficient in leptin or the leptin receptor to determine whether leptin contributes to arterial thrombosis following endothelial injury.

METHODS

Mice

All mice used for these experiments, which were performed between September 2000 and September 2001, were purchased from The Jackson Laboratory, Bar Harbor, Me. Leptin-deficient (*ob/ob*),⁷ leptin receptor-deficient (*db/db*),⁸ and

Context Complications of atherosclerosis are the leading cause of morbidity and mortality in industrialized societies. Obesity has emerged as an independent risk factor for complications of atherosclerotic vascular disease. Leptin, a hormone produced by the adipocyte, increases with obesity and appears to modulate energy balance and food intake. In addition, other actions of leptin have been proposed, including an *in vitro* effect on platelet aggregation. Thus, the elevated plasma leptin levels in obese individuals may promote vascular thrombosis.

Objective To test the hypothesis that leptin contributes to *in vivo* thrombosis via the leptin receptor.

Design and Materials Between September 2000 and September 2001, a vascular thrombosis model was used to test male 10- to 12-week-old mice completely deficient in leptin or the leptin receptor and mice with platelet leptin-receptor deficiency.

Main Outcome Measure Time to formation of an occlusive thrombus in the common carotid artery following experimentally induced endothelial injury.

Results Following onset of vascular injury, wild-type mice (*n*=8) formed occlusive thrombosis in a mean (SD) of 42.2 (4.6) minutes, whereas leptin-deficient (*n*=5) and leptin receptor-deficient mice (*n*=7) formed occlusive thrombosis in 75.2 (10.1) and 68.6 (10.3) minutes, respectively (leptin deficient vs wild-type mice, *P*=.008; leptin-receptor-deficient vs wild-type, *P*=.03). When recombinant murine leptin was administered to leptin-deficient mice (*n*=4), the time to occlusion was reduced to 41.8 (6.6) minutes (*P*=.035 vs vehicle control). Following bone marrow transplantation from leptin receptor-deficient (donor) mice to wild-type (recipient) mice, the time to occlusion was prolonged from 22.3 (2.8) minutes in wild-type mice receiving wild-type marrow (*n*=3) to 56.8 (5.0) minutes in wild-type mice receiving leptin receptor-deficient bone marrow (*n*=5) (*P*=.003).

Conclusions Leptin contributes to arterial thrombosis following vascular injury *in vivo* and these prothrombotic effects appear to be mediated through the platelet leptin receptor.

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wild-type mice were of the C57BL/6J strain. All mice were male and between 10 and 12 weeks of age. They were fed normal chow in specific pathogen-free facilities. All animal care and experimental procedures complied with the Principles of Laboratory and Animal Care established by the National Society for Medical Research and were approved by the University of Michigan Committee on Use and Care of Animals.

Arterial Thrombosis Protocol

Carotid arterial injury and thrombosis were induced with a photochemical in-

jury model as described previously.⁹ This model causes endothelial damage at the site of the photochemical injury, followed by platelet aggregation and the formation of a platelet- and fibrin-rich thrombus. Briefly, the mice were anesthetized with intraperitoneal sodium pentobarbital (Butler Co, Columbus, Ohio). Mice were then se-

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cured in the supine position and placed under a dissecting microscope (Nikon SMZ-2T, Mager Scientific Inc, Dexter, Mich). After a midline cervical incision, the right common carotid artery was isolated, and a Doppler flow probe (model 0.5 VB, Transonic Systems, Ithaca, NY) was applied. The probe was connected to a flow meter (Transonic model T106, Transonic Systems) and interpreted with a computerized data acquisition program (Windaq, DATAQ Instruments, Akron, Ohio). The photochemical rose bengal (Fisher Scientific, Fair Lawn, NJ) was diluted to 10 mg/mL in phosphate-buffered saline and then injected into the tail vein in a volume of 0.12 mL at 50 mg/kg by use of a 27-gauge Precision Glide needle with a 1-mL latex-free syringe (Becton Dickinson and Co, Franklin Lakes, NJ). To activate the rose bengal, a 1.5-mW green light laser (540 nm, Melles Griot, Carlsbad, Calif) was applied to the mid common carotid artery from a distance of 6 cm. Flow in the vessel was monitored continuously from the time of the injection until the end of the experiment. The end point for these studies was determined by 2 criteria: the cessation of flow in the carotid artery as measured by the Doppler flow probe and the visualization of an occlusive thrombus via a dissecting microscope by an observer blinded to the treatment group.

Leptin Administration

Recombinant murine leptin (Research Diagnostics Inc, Flanders, NJ) was reconstituted according to the manufacturer's instructions and kept frozen until immediately before administration. Recombinant leptin (9 µg per injection) was administered to leptin-deficient and leptin receptor-deficient mice via intraperitoneal injection 10 minutes before and 10 minutes after the intravenous injection of the rose bengal. With the same protocol, vehicle control injections were given to leptin-deficient mice. A commercially available assay (Crystal Chem Inc, Chicago, Ill) was used according to the manufacturer's instructions to de-

termine plasma leptin levels in duplicate from platelet-poor plasma.

Bone Marrow Transplantation

Bone marrow transplantation experiments were performed with methods similar to those previously described.¹⁰ Briefly, 8-week-old male wild-type mice were used as recipients for bone marrow transplantation from wild-type and leptin receptor-deficient mice. Bone marrow was harvested from the donor mice by flushing their femurs and tibias with RPMI 1640 medium containing 2% fetal bovine serum. Cells were then centrifuged at 300g and resuspended in phosphate-buffered saline before injection. Each recipient mouse was irradiated (2×650 rad [0.02×6.5 Gy]) and injected with 4×10^6 bone marrow cells via the tail vein. Four weeks after transplantation, blood was withdrawn from the retro-orbital sinus, and platelet counts were determined by using platelet-rich plasma in Coulter Counter Z2 (Beckman Coulter, Miami, Fla) according to the manufacturer's instructions. The arterial thrombosis protocol was performed the following day.

Histologic Analyses

For some mice, 3 wild-type and 3 leptin deficient, the arterial vasculature was perfused with formalin immediately after the thrombosis protocol was completed, and the common carotid artery at the site of the occlusive thrombus was excised, sectioned, and subjected to hematoxylin-eosin staining.

Statistical Analysis

The analysis of differences in time to occlusion was determined with the *t* test; $P < .05$ was considered statistically significant (SigmaStat version 2.03, SPSS Science, Chicago, Ill).

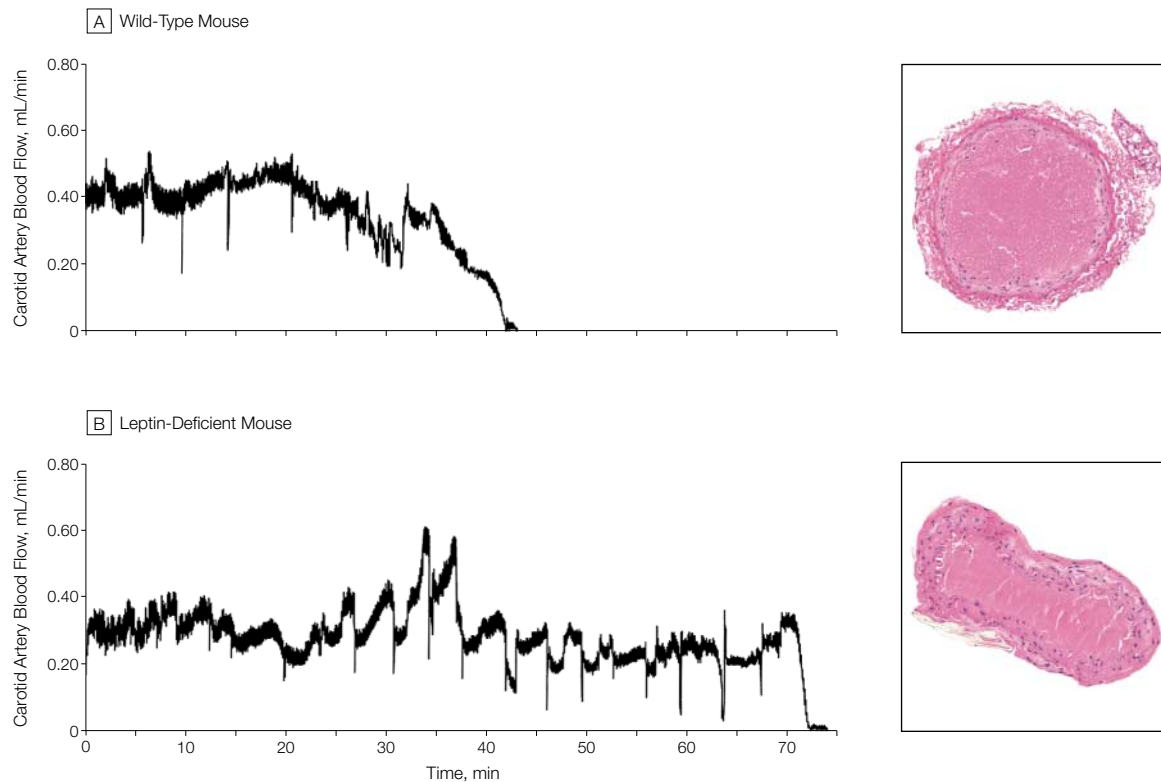
RESULTS

To examine the influence of leptin and the leptin receptor on in vivo thrombosis, we examined mice deficient in leptin ($n=5$) or the leptin receptor ($n=7$) in a photochemical injury model of arterial thrombosis. Compared with

wild-type mice ($n=8$), leptin-deficient mice had a prolonged time to occlusion following photochemical injury (FIGURE 1) and FIGURE 2), as did leptin receptor-deficient mice (Figure 2).

To determine whether the phenotype of the leptin-deficient mice was due to the lack of leptin, we administered recombinant murine leptin during the photochemical injury. In leptin-deficient mice ($n=4$), this treatment resulted in a significant reduction in the time to occlusive thrombosis, which was similar to the occlusion times observed in wild-type mice (Figure 2). Occlusion times in mice lacking the leptin receptor ($n=4$) were not affected by recombinant murine leptin (Figure 2) ($P=.90$). Blood samples obtained immediately after the arterial thrombosis experiments revealed plasma leptin levels between 50 and 750 ng/mL, with a mean plasma leptin concentration of 299 ng/mL. Normal physiological leptin levels in mice range from 1 to 170 ng/mL.¹¹

To examine whether the leptin receptor-deficient phenotype was due to the lack of the receptor on the endothelial cells or the platelet, we performed bone marrow transplantations from leptin receptor-deficient mice to wild-type mice. With this procedure, mice that underwent successful transplantations would be deficient for the leptin receptor only in bone marrow-derived cells, allowing specific assessment of address the platelet leptin receptor. Four weeks after the bone marrow transplantation, the mean platelet counts between mice that received either wild-type ($n=3$; $390 \times 10^3/\mu\text{L}$) or leptin receptor-deficient bone marrow ($n=5$; $321 \times 10^3/\mu\text{L}$) were similar. In addition, mean (SD) plasma leptin levels were not significantly different between control (1.3 [0.1] ng/mL) and leptin receptor-deficient (1.6 [0.5] ng/mL) bone marrow recipients ($P=.65$). Mice that had received leptin receptor-deficient bone marrow ($n=5$) had a significantly longer mean (SD) time to occlusive thrombosis (56.8 [5.0] minutes) compared with wild-type mice ($n=3$) that had received wild-type bone marrow (22.3 [2.8] minutes; $P=.003$).

Figure 1. Representative Compressed Carotid Artery Blood Flow Tracings and Cross Sections

Compressed carotid artery blood flow tracings from a wild-type (A) and a leptin-deficient (B) mouse from the initiation of injury until the cessation of blood flow (occlusive thrombosis) are shown. The downward deflections that precede total occlusion represent transient blood-flow reductions caused by episodic subocclusive thrombosis. Figures on the right are representative cross sections of the occlusive thrombus stained with hematoxylin-eosin (original magnification $\times 200$). There were no apparent differences in the appearance of the cross sections.

A control mouse that did not receive bone marrow following irradiation died 9 days later, demonstrating effective bone marrow ablation following the irradiation.

COMMENT

Thrombotic complications of vascular disease are the leading cause of morbidity and mortality in industrialized societies. Although obesity is now considered an independent risk factor for cardiovascular disease,¹ the mechanisms that link obesity to cardiovascular disease risk are unclear.

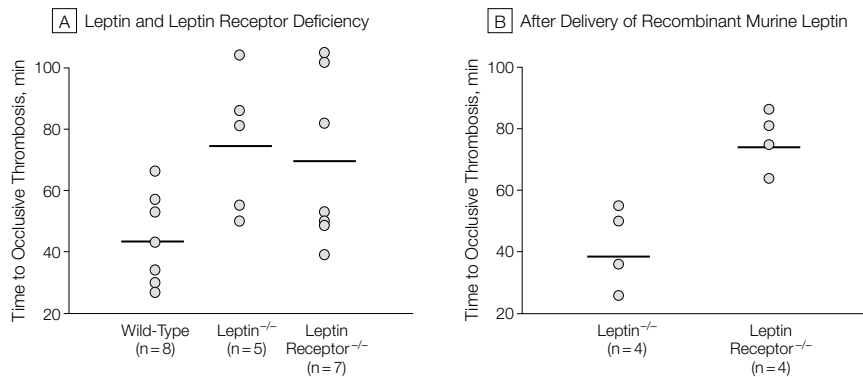
Studies have demonstrated associations of obesity with enhanced expression of factors that may favor thrombosis, including plasminogen activator inhibitor type 1,^{12,13} tissue factor,⁵ and tumor necrosis factor α .¹⁴ However, in vivo data demonstrating a causal rela-

tionship with obesity-related gene expression changes and thrombosis have not been established.

The recent identification of a long-form functional leptin receptor (OB-Rb) on the human platelet suggests there might be signaling between the adipocyte and the platelet.¹⁵ This interaction between leptin and the platelet was supported by the dose-dependent increase in *in vitro* platelet aggregation in the presence of a platelet agonist. The platelet-stimulating effect was shown to be closely linked with the phosphorylation of platelet protein tyrosine residues. The tyrosine phosphorylation and platelet aggregation were demonstrated at relatively high levels of leptin, suggesting that the interaction between leptin and the platelet would be most relevant in obese and diabetic individuals in whom leptin levels are elevated.¹⁵

Although leptin has been demonstrated to affect food intake and metabolism, it may also affect many other processes.¹⁶ In this report, by using an experimental model that is sensitive to changes in coagulation¹⁷ and fibrinolytic⁹ factors, we have demonstrated that the absence of leptin or the leptin receptor has an important influence on *in vivo* thrombosis. Furthermore, we provide evidence that the short-term addition of leptin reverses the antithrombotic phenotype of the leptin-deficient mice but not the leptin receptor-deficient mice, demonstrating the receptor-dependent effect of leptin. Moreover, these findings suggest that the absence of the platelet leptin receptor following the bone marrow transplantation resulted in prolongation of the time to occlusion in wild-type mice with relatively low plasma leptin levels. Although

Figure 2. Effect of Leptin and the Leptin Receptor on Time to Occlusive Arterial Thrombosis Following Photochemical Injury



A, Mice deficient in leptin or the leptin receptor had a significantly prolonged time to occlusive thrombosis compared with wild-type mice ($P=.008$). B, In leptin-deficient mice, recombinant murine leptin significantly shortened the time to occlusive thrombosis ($P=.04$) compared with leptin-deficient mice that received vehicle control. There was no effect of recombinant murine leptin on thrombosis in leptin receptor-deficient mice ($P=.90$).

we cannot exclude an effect from other bone marrow-derived cells, the platelet-rich nature of the thrombus in this model suggests that the interaction between circulating leptin and the platelet leptin receptor is highly sensitive and physiologically important even at low leptin levels. Therefore, the interaction between leptin and the leptin receptor may affect platelet aggregation and thrombosis in both lean and obese individuals. In addition, after our study was conducted, Konstantinides et al¹⁸ published results describing a different thrombosis model with a similar leptin effect.

These studies provide a potential link between obesity and cardiovascular disease risk. Wallace et al¹⁹ demonstrated that leptin is an independent risk factor for coronary events. In their case-control study of hypercholesterolemic men, a 1-SD increase in leptin was associated with a 20% increase in risk for coronary events. Although further clinical studies are necessary to determine the magnitude of the cardiovascular risk associated with elevated leptin levels, these findings raise the possibility that therapeutic strategies aimed at reducing plasma leptin levels may reduce cardiovascular events. However, the potential beneficial effects must be

weighed against the adverse effects that may arise with drug-induced leptin reduction, such as weight gain and its associated comorbidities. Since leptin levels correlate well with adiposity, strategies aimed at weight reduction should remain first-line therapies. Nevertheless, a strategy specifically targeting the leptin receptor on the platelet could provide benefit independent of changes in leptin.

In conclusion, we have demonstrated that leptin and the leptin receptor affect arterial thrombosis following endothelial injury and that these effects appear to be mediated by an interaction between leptin and the platelet leptin receptor. These findings provide a potential link between increasing adiposity and thrombotic risk.

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REFERENCES

- Eckel RH, Krauss RM, for the AHA Nutrition Committee. American Heart Association call to action: obesity as a major risk factor for coronary heart disease. *Circulation*. 1998;97:2099-2100.
- Fuster V, Stein B, Ambrose JA, et al. Atherosclerotic plaque rupture and thrombosis: evolving concepts. *Circulation*. 1990;82(suppl 3):II47-II59.
- Juhan-Vague I, Alessi MC, Morange PE. Hypofibrinolysis and increased PAI-1 are linked to atherothrombosis via insulin resistance and obesity. *Ann Med*. 2000;32(suppl 1):78-84.
- Winkler UH. Obesity and haemostasis. *Arch Gynecol Obstet*. 1997;261:25-29.
- Samad F, Pandey M, Loskutoff DJ. Tissue factor gene expression in the adipose tissues of obese mice. *Proc Natl Acad Sci U S A*. 1998;95:7591-7596.
- Nakata M, Yada T, Soejima N, Maruyama I. Leptin promotes aggregation of human platelets via the long form of its receptor. *Diabetes*. 1999;48:426-429.
- Halaas JL, Gajiwala KS, Maffei M, et al. Weight-reducing effects of the plasma protein encoded by the obese gene. *Science*. 1995;269:543-546.
- Chen H, Charlat O, Tartaglia LA, et al. Evidence that the diabetes gene encodes the leptin receptor: identification of a mutation in the leptin receptor gene in db/db mice. *Cell*. 1996;84:491-495.
- Eitzman DT, Westrick RJ, Nabel EG, Ginsburg D. Plasminogen activator inhibitor-1 and vitronectin promote vascular thrombosis in mice. *Blood*. 2000;95:577-580.
- Linton MF, Atkinson JB, Fazio S. Prevention of atherosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation. *Science*. 1995;267:1034-1037.
- Ishizuka T, Klepcyk P, Liu S, et al. Effects of overexpression of human GLUT4 gene on maternal diabetes and fetal growth in spontaneous gestational diabetic C57BLKS/J Lepr(db/+) mice. *Diabetes*. 1999;48:1061-1069.
- Samad F, Yamamoto K, Loskutoff DJ. Distribution and regulation of plasminogen activator inhibitor-1 in murine adipose tissue in vivo. *J Clin Invest*. 1996;97:37-46.
- Lundgren CH, Brown SL, Nordt TK, Sobel BE, Fujii S. Elaboration of type-1 plasminogen activator inhibitor from adipocytes: a potential pathogenetic link between obesity and cardiovascular disease. *Circulation*. 1996;93:106-110.
- Samad F, Uysal KT, Wiesbrock SM, et al. Tumor necrosis factor alpha is a key component in the obesity-linked elevation of plasminogen activator inhibitor 1. *Proc Natl Acad Sci U S A*. 1999;96:6902-6907.
- Maruyama I, Nakata M, Yamaji K. Effect of leptin in platelet and endothelial cells: obesity and arterial thrombosis. *Ann N Y Acad Sci*. 2000;902:315-319.
- Fantuzzi G, Faggioni R. Leptin in the regulation of immunity, inflammation, and hematopoiesis. *J Leukoc Biol*. 2000;68:437-446.
- Westrick RJ, Bodary PF, Xu Z, Shen YC, Broze GJ, Eitzman DT. Deficiency of tissue factor pathway inhibitor promotes atherosclerosis and thrombosis in mice. *Circulation*. 2001;103:3044-3046.
- Konstantinides S, Schafer K, Koschnick S, Loskutoff DJ. Leptin-dependent platelet aggregation and arterial thrombosis suggests a mechanism for atherothrombotic disease in obesity. 2001;108:1533-1540.
- Wallace AM, McMahon AD, Packard CJ, et al. Plasma leptin and the risk of cardiovascular disease in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation*. 2001;104:3052-3056.