Thrombosis

Inhibition of Endogenous Leptin Protects Mice From Arterial and Venous Thrombosis

Stavros Konstantinides, Katrin Schäfer, Jaap G. Neels, Claudia Della, David J. Loskutoff

Objective—Human obesity is associated with an increased incidence of cardiovascular disease and, in particular, with an elevated risk for arterial and venous thrombosis.1–3 Excess body weight and the accompanying metabolic disorders may contribute to the development of a systemic prothrombotic state by altering the balance between thrombotic and thrombolytic activity in the circulation.4 In this regard, human and murine obesity are associated with increased circulating levels of a variety of antifibrinolytic and prothrombotic factors, including plasminogen activator inhibitor-1 and tissue factor, and it is clear that these molecules may be produced by the adipocytes themselves.5,6 However, despite the high plasminogen activator inhibitor-1 and tissue factor levels, leptin-deficient ob/ob mice showed an attenuated, rather than enhanced, thrombotic response to arterial injury, and they exhibited a defect in platelet aggregation when compared with lean wild-type (WT) littermates.7,8 These and other9,10 observations suggest that leptin, the 167-amino acid polypeptide that is encoded by the ob gene and regulates food intake and body weight,11 may also modulate platelet function and thrombosis. Because the ob/ob mouse lacks functional leptin at birth, it accumulates an excess of adipose tissue at an early age, and type II diabetes and other features of the metabolic syndrome develop. In contrast to the situation in ob/ob mice, human obesity is frequently characterized by leptin resistance and elevated plasma levels of the hormone.11,12

In the present study, we used the ferric chloride (FeCl3) model of arterial injury and thrombosis7 and the collagen–epinephrine model of venous thromboembolism13 to study the effects of endogenous leptin on thrombosis in the mouse. Instead of using ob/ob mice with their complex phenotype, or of artificially raising the circulating leptin levels in mice using exogenous recombinant murine leptin,7,8 we administered a leptin-neutralizing antibody to lean mice to selectively but transiently block the binding of endogenous circulating leptin to its receptor. We demonstrate that inhibition of circulating endogenous leptin not only reduces thrombus size but also protects lean mice from experimentally induced arterial and venous thrombosis in vivo. Thus, endogenous leptin may represent a new target for treating cardiovascular disease in obesity.

Methods

Animals

WT (C57BL/6J) mice were from the breeding colony of the Scripps Research Institute, and ob/ob (C57BL/6J-Lepob) mice were purchased from Jackson Laboratories (Bar Harbor, Me). Mice (6- to

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10-week-old) were anesthetized by inhalation of methoxyflurane (Metofane; Schering-Plough, Union, NJ), and then treated as described. All animal care and experimental procedures were approved by the Animal Research Committee of the Scripps Research Institute and complied with the Guide for Care and Use of Laboratory Animals, Department of Health, Education, and Welfare.

Inhibition of Leptin Binding to Its Receptor

The ability of a polyclonal affinity-purified goat IgG developed against recombinant mouse leptin (AF-498; R&D Systems, Minneapolis, Minn) to block the binding of leptin to its receptor in vitro was determined by surface plasmon resonance technology, essentially as described. Briefly, a bovine serum albumin-free recombinant mouse leptin receptor/Fc chimera (Research Diagnostics Inc, Flanders, NJ) was immobilized onto a CM5-sensorchip, using the amine coupling kit as prescribed by the supplier (BIAcore). A control channel was activated and blocked in the absence of protein. Binding to coated channels was corrected for binding to noncoated channels (<5% of binding to coated channels). Murine leptin (Calbiochem) at a concentration of 100 nM was passed over the sensorchip surface either alone or after 15 minutes of preincubation at room temperature with increasing concentrations of the antibody. As a control, 1.2 μmol/L of antibody alone was passed over the sensorchip surface. The BIAevaluation (version 3.1) software was used to evaluate the resulting binding curves. The maximal leptin binding response, corrected for bulk refractive index changes and nonspecific binding, was expressed as percentage residual binding, with binding of 100 nM leptin alone set at 100%. The resulting data were plotted using GraphPad Prism software version 3.02, and the best fit was obtained using a quadratic equation model. Regeneration of the sensorchip surface was performed by incubation with 100 mmol/L H3PO4 for 2 minutes at a flow of 5 μL/min.

The effect of the antibody to block leptin to its receptor in vivo was also determined. Because the circulating levels of leptin in the plasma of WT mice were close to the detection level of our assay, it was necessary to “spike” the plasma with exogenous leptin. This was accomplished by injecting 1 μg of leptin intraperitoneally into WT mice 30 minutes before the injection of a nonspecific goat IgG (Sigma, St. Louis, Mo) or the neutralizing IgG (total volume 75 μL) into the right jugular vein. This treatment raised the concentration of circulating leptin by ~5-fold (ie, from 2 to 10 ng/mL). Fifteen minutes later, blood was collected by cardiac puncture and plasma (400 μL) was prepared and incubated for 15 minutes at room temperature with soluble leptin receptor bound to CNBr-activated Sepharose 4 Fast Flow beads. The bound leptin was cross-linked to its receptor by DTSSP (0.5 mmol/L; Pierce), washed 3 times, separated under reducing conditions by SDS-PAGE on a 4% to 20% gel, transferred to polyvinylidene fluoride (PVDF) membrane, and separated with polyclonal anti-mouse leptin antibody. The leptin detected in these pull-down experiments migrated with the expected Mr of 16 kDa. The soluble leptin receptor used for these studies (murine) was expressed as a His-tagged fusion protein in stably transfected S2 insect cells using the pMT/BI/VP5-His vector and the DES Inducible/Secreted Kit (Invitrogen) according to the manufacturer’s instructions. It was purified by homogeneity by affinity chromatography using recombinant mouse leptin (4 mg) coupled to a 1-mL HiTrap NHS-activated HP column (Amersham Biosciences) according to the manufacturer’s instructions, and then coupled to sepharose beads as described.

FeCl3-Induced Arterial Injury

Anesthetized WT mice were subjected to carotid artery injury using 10% FeCl3 according to a standardized protocol. In some instances, 3.0 μg of either the leptin-neutralizing antibody or the nonspecific goat IgG was injected into the right jugular vein of the mice (n=14 each) 15 minutes before injury. Carotid blood flow was monitored before and during the 25-minute interval after injury using an ultrasound flow probe interfaced with a flowmeter and a data acquisition program. The investigator performing the experiments was unaware of the identity of the injected IgG.

Pulmonary Embolism Induced by Injection of Collagen and Epinephrine

Venous thromboembolism was induced in WT and ob/ob mice using a recently described protocol. Briefly, the right jugular vein of anesthetized mice was surgically exposed and a mixture of equine tendon type I collagen (0.5 mg/kg; Chrono-Log Corporation, Havertown, Pa) plus epinephrine (60 μg/kg; Sigma) was infused into the vein over a 5-second interval using an insulin syringe. Time to death was defined as the time to respiratory arrest lasting at least 1 minute. Preliminary studies (not shown) revealed that death always occurred within the first 10 minutes, and that animals that survived beyond this time recovered fully.

Histochemical Analysis of Mouse Lungs

To quantify the number of thrombi in the pulmonary vasculature, 7 WT and 7 ob/ob mice were euthanized by cervical dislocation 3 minutes after injection of the collagen–epinephrine mixture, and their lungs were excised and formalin-fixed. Paraffin sections (5-μm-thick) were stained with Masson trichrome stain and then analyzed histologically for the presence of thrombi. For each mouse, 8 fields (magnification, 200×) were chosen at random from within the left lobe, and those vessels larger than 50 μm in diameter that contained occlusive thrombi were counted. The average number of occluded vessels per field was then calculated for each animal. Immunohistochemistry also was performed on the paraffin-embedded lung sections as described. Platelets (and endothelial cells) were identified using a rabbit anti-human von Willebrand factor antibody (Dako, Hamburg, Germany; dilution 1:100). Fibrinogen/fibrin was detected as described using a rabbit anti-human fibrinogen antibody (Dako; dilution 1:200).

Statistical Analysis

Differences between medians were tested by the Mann–Whitney nonparametric test. In these analyses, mice that did not reach the end point (arterial occlusion after injury with FeCl3, or death from venous thromboembolism) were defined as having times to the end point that corresponded to the end of the monitoring period. For all other continuous variables, differences between mean values were tested by the Student t test. Qualitative variables were tested by Fisher exact test. All statistical tests were 2-sided, with P<0.05 indicating statistically significant difference.

Results

The Leptin Neutalizing Antibody Inhibits the Binding of Leptin to Its Receptor

The ability of the affinity-purified antibody to inhibit the interaction between leptin and the leptin receptor was initially determined under in vitro conditions using surface plasmon resonance technology. A sharp, dose-dependent decrease in binding of leptin to its receptor in vivo (Figure 1, inset). The injection of the neutralizing IgG into mice also inhibited the effects of this inhibition on arterial thrombosis were tested. As expected, treatment of carotid arteries from WT mice
with FeCl₃ resulted in the complete cessation of flow (Figure 2A), indicating the formation of stable occlusive thrombi. However, when the WT mice were pretreated with the leptin-neutralizing antibody, the resulting thrombi were unstable and frequently embolized (Figure 2B). In this instance, considerable flow was still observed at the end of the recording period (Figure 2B), and the median time to complete thrombotic occlusion (Figure 3A) was significantly prolonged compared with mice pretreated with the control IgG (9.8 versus 7.4 minutes; *P* < 0.004). Moreover, at the end of the experiment (25 minutes after injury), the proportion of patent vessels increased from ~7% in mice pretreated with nonspecific IgG to >50% in mice that received the leptin-neutralizing antibody (Figure 3B; *P* < 0.05).

**Effects of Leptin on Venous Thromboembolism**

The contribution of endogenous leptin to venous thromboembolism was examined using the collagen–epinephrine model. Less than 10% of WT mice pretreated with the nonspecific IgG survived after injection of the collagen–epinephrine mixture into the jugular vein (Figure 4A). However, ~60% of mice survived if they had been pretreated with the neutralizing antibody. Thus, intravenous administration of the leptin-neutralizing antibody before the injection of the collagen/epinephrine mixture appeared to protect WT mice from lethal venous thrombosis and pulmonary embolism. The fact that the leptin-deficient ob/ob mice were also protected (Figure 4B) emphasizes the critical role of endogenous leptin in venous thrombosis in this model.

Histochemical analysis of mouse lungs harvested 3 minutes after collagen–epinephrine injection showed the presence of occlusive thrombi in vessels from both WT and ob/ob mice (Figure 5). The pulmonary thrombi that formed in both strains were rich in platelets (Figure 5A and 5B) and fibrinogen/fibrin (Figure 5C and 5D), and there was little apparent difference in composition between them. However, quantitative analysis of sections stained with Masson trichrome stain revealed that thrombosis was significantly more pronounced in lung vessels from WT mice versus those from ob/ob mice (Figure 5E). For example, occlusive thrombi were detected in 12.7±1.0 pulmonary vessels (>50 μm in diameter) per field in the WT mice compared with 8.6±1.1 vessels per field in the ob/ob mice (*P* = 0.007). Although not shown, the number of occlusive thrombi was also significantly decreased (*P* = 0.05) in vessels from WT mice treated with the leptin-neutralizing IgG (7.6±2.3 pulmonary vessels...
per field; n=5) compared with their WT counterparts treated with the nonspecific IgG (11.8±3.1 vessels per field; n=5).

Discussion

The biological spectrum of the adipocyte hormone leptin is now known to extend far beyond the regulation of food intake and body weight.11 In particular, accumulating evidence suggests that leptin may represent a novel direct link between obesity and cardiovascular disease.18,19 For example, leptin exerts pleiotropic effects on homeostasis of the vessel wall by increasing blood pressure,20 and possibly by modulating the function of endothelial cells,21 macrophages,22 and vascular smooth muscle cells.23 Recently, leptin was shown to promote vascular remodeling and neointimal growth after injury in mice,24 and to enhance platelet aggregation and arterial thrombosis in vivo.7–10 Leptin-deficient ob/ob mice demonstrate an attenuated thrombotic response to arterial injury compared with their lean WT counterparts,7,8 and leptin administration promotes thrombosis in this model. Importantly, the effects of leptin on thrombosis appear to be mediated by the long form of the leptin receptor because exogenous leptin does not enhance thrombosis in obese db/db mice,7,8 which lack the functional isoform of this receptor.11

In the present study, we demonstrate that intravenous administration of a leptin-neutralizing antibody into WT mice before arterial injury with FeCl3 inhibited the binding of leptin to its receptor (Figure 1) and prolonged the time to thrombotic occlusion (Figure 3A). This treatment also led to a dramatic increase in the rate of vascular patency (Figure 3B), primarily because of the formation of unstable, frequently embolizing thrombi (Figure 2). Thus, inhibition of endogenous leptin attenuated the thrombotic response of WT mice. The potential deleterious effects of reduced thrombus stability (eg, stroke, death) were not observed here or in previous long-term studies.25

Apart from its association with an increased risk for arterial thrombosis, obesity may also be a risk factor for venous thromboembolism in humans.3 Because platelets express the long form of the leptin receptor,10 and because platelet activation contributes to the formation of both arterial and venous thrombi,26 we hypothesized that inhibition of circulating leptin in mice might also result in resistance to venous thromboembolism. To test this hypothesis, we examined the effects of the leptin-neutralizing antibodies on the occurrence of fatal pulmonary embolism induced in WT mice by injection of a mixture of collagen and epinephrine into the jugular vein. Collagen and epinephrine are well-known agonists of platelet activation. Although this model does not exactly reproduce the complex pathophysiology of venous thrombosis and pulmonary embolism in humans, it has been used successfully for studying defects in platelet activation and their effects on systemic intravascular thrombosis in vivo.13,27 As expected,13 injection of collagen plus epinephrine rapidly induced lethal pulmonary embolism in the majority of WT mice treated with the control (nonspecific) IgG (Figure 4A). However, if the WT mice were pretreated with the leptin-neutralizing antibody, they were partially protected from these effects. For example, 60% of the mice that received the antibody survived, compared with only 9% of those treated with nonspecific IgG. In fact, the survival curves for the antibody-treated WT mice were very similar to those obtained using the ob/ob mice (Figure 4B versus 4A). Thus, inhibition and/or lack of leptin protect mice from pulmonary embolism-related death in this model. The differences in survival in both cases were associated with differences in the extent of thrombotic vascular occlusion in the mouse lungs.
The mechanisms that mediate the effects of leptin on platelet aggregation and thrombosis are not understood. By binding to the long form of the leptin receptor on human and murine platelets, leptin may induce tyrosine phosphorylation of platelet proteins via the JAK/STAT pathway, possibly amplifying the intracellular signals generated from the ADP and thrombin receptors.6,9,10 These effects of leptin probably occur upstream of phospholipase C activation and intracellular calcium mobilization.9 In this regard, it is interesting to note that a number of other cytokines, including thrombopoietin,28 Gas6,13 and CD40L,29 also potentiate platelet aggregation and secretion in response to platelet agonists. Furthermore, and in accordance with these findings, the absence of the Gas613 or CD40L29 genes protected mice from injury-induced thrombosis in vivo. Thus, the phenotypes of mice lacking Gas6, CD40L, and possibly thrombopoietin, resemble that of mice lacking functional leptin, whether genetically induced (ie, in ob/ob mice) or induced experimentally by infusion of neutralizing antibodies. Although these genes all seem to modify platelet function and thrombosis in the mouse, it is not yet clear whether they do so by acting on common pathways or through distinct mechanisms.

The ob/ob mouse is a powerful model for the study of human obesity. However, although ob/ob mice exhibit several metabolic abnormalities that resemble those comprising the metabolic syndrome in humans,6,11 atherosclerosis or thrombosis does not spontaneously develop.10 One important difference is that ob/ob mice lack functional leptin,11 in contrast to the hyperleptinemia encountered in the majority of obese humans.11,31 This difference may be critical because the results of the present study suggest that increased levels of leptin in the circulation of obese humans12 may contribute to the elevated risk for thrombotic complications associated with this condition.19,32

In conclusion, the present study shows that transient inhibition of endogenous leptin attenuates the arterial thrombotic response to injury in lean mice and also protects lean mice from lethal venous thromboembolism. The neutralizing antibody does so without promoting obesity or type II diabetes, common features of the chronic leptin deficiency associated with ob/ob mice.11 These observations strengthen the hypothesis that leptin is an important regulator of arterial and venous thrombosis in vivo and suggest that these effects may depend on the binding of leptin to its receptor on platelets. Further studies are now needed to clarify the exact mechanisms and pathways by which leptin modulates platelet function and also to determine whether inhibition of the prothrombotic effects of leptin may represent a novel therapeutic strategy for reducing the cardiovascular risk associated with excess body weight in humans.

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References


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