Effect of Leptin on Vascular Calcification in Apolipoprotein E–Deficient Mice

Melec Zeadin, Martin Butcher, Geoff Werstuck, Mohammad Khan, Colin K. Yee, Stephen G. Shaughnessy

Objective—The adipocytokine leptin has been proposed to increase cardiovascular risk in both obese and diabetic individuals. In the current study, therefore, we used apoE-deficient mice to examine the effects of leptin on both lesion size and calcification.

Methods and Results—Mice were treated with once daily intraperitoneal injections of leptin (125 μg/mouse/d) for 2 months. The mice were then euthanized, and sections of the aortic root and thoracic aorta analyzed histomorphometrically. Measurements of lesion size and surface area occupied by atherosclerotic lesions did not reveal any differences between nontreated and leptin-treated animals. However, von Kossa staining of the aortic root demonstrated an 8.3±2.0-fold increase in lesion calcification as well as a 2.5±0.6-fold increase in valvular calcification in those animals treated with leptin. In addition, the percent total lesion area demonstrating ALP-positive staining was 5.4±2.1-fold greater in leptin-treated mice when compared to nontreated control mice. This increase in ALP staining was also accompanied by an increase in the expression of the osteoblast-specific markers, osteocalcin, and osteopontin.

Conclusions—Based on these observations, we conclude that leptin may increase cardiovascular risk by promoting osteogenic differentiation and thus vascular calcification. (Arterioscler Thromb Vasc Biol. 2009;29:2069-2075.)

Key Words: leptin ■ vascular calcification ■ atherosclerosis ■ osteoblasts ■ valvular stenosis

Recent studies suggest that factors secreted by adipose tissue may be playing a role in promoting cardiovascular disease (CVD). One such group of factors, referred to as adipocytokines, is known to circulate at elevated levels in obese individuals and to positively correlate with an increased risk of CVD.1,2 In particular, clinical studies have shown that high circulating levels of the adipocytokine leptin can accelerate the atherosclerotic process and is thus predictive of adverse cardiovascular events in both obese and diabetic populations.3,4 The possible proatherogenic effects of leptin include induction of endothelial cell dysfunction, stimulation of inflammatory processes, increased levels of oxidative stress, and increased migration and proliferation of vascular smooth muscle cells.5–8

Leptin has been shown to promote neointimal thickening after chemical or mechanical injury9 and to promote lesion development in apoE-deficient mice.10,11 In addition, Parhami et al demonstrated that leptin can induce vascular smooth muscle cells to undergo a phenotypic transition into bone-forming cells, which are capable of mineralization in vitro.12 When taken together, these findings suggest that leptin may be acting to increase the risk of myocardial infarction by promoting not only lesion development but also vascular calcification in vivo.

The clinical consequences of vascular calcification are well documented. For example, arterial calcification is known to increase vessel wall rigidity,13 decrease diastolic coronary perfusion,14,15 and increase the risk of myocardial infarction or stroke.16–18 Furthermore, calcified atherosclerotic plaques are known to be less stable and thus more likely to cause vessel wall dissection during balloon angioplasty or stent placement.19–22 Finally, the calcification of cardiac valves can lead to valvular aortic stenosis resulting in a loss of valve mobility.23,24

In the current study, we use an established animal model of atherosclerosis to examine the effect of leptin on vascular calcification. Herein, we report that elevated levels of leptin can promote vascular calcification in vivo. In addition, by measuring the expression of osteoblast-specific markers within the lesions of leptin-treated mice, we were able to demonstrate that increased vascular calcification is associated with an increase in osteoblast number.

Materials and Methods

Materials

Recombinant murine leptin was purchased from R&D Systems, and triglyceride and cholesterol measuring reagents were obtained from ThermoFisher Scientific. Alkaline phosphatase kits were purchased...
from Sigma-Aldrich, whereas all antibodies were obtained from Santa Cruz Biotechnology Inc. Finally, the DIG-RNA Labeling Kit, Blocking Reagent, anti-Digoxigenin-AP antibody, and NBT/BCIP Ready-to-Use tablets were purchased from Roche Diagnostics. All other reagents for in situ hybridization experiments were purchased from Sigma-Aldrich.

**Experimental Protocol**

Beginning at 8 weeks of age, female apoE-deficient mice were placed on a high-fat Western diet (Harlan Tekland; TD. 881173) and then randomized into 1 of 2 treatment groups. The first group (n=7 animals per group) received daily intraperitoneal (i.p.) injections of recombinant murine leptin at a concentration of 125 μg/mouse, whereas the second (n=7 animals) received an equivalent volume of saline (200 μL i.p./mouse) and acted as the vehicle control. On day 60, all animals were euthanized, and the heart, including the thoracic aorta, was removed for histological evaluation.

**Tissue Sample Preparations and Histology**

On day 60, all animals were fasted for 4 hours to obtain fasting blood glucose levels using a glucometer (Bayer). Whole blood was also collected into K3-EDTA-coated microtubes (VWR) via cardiac puncture of the right ventricle. Plasma lipid levels and insulin levels were determined using colorimetric diagnostic kits for total cholesterol/triglycerides and plasma insulin (Crystal Chem Inc). After the animals were euthanized, the intact circulation was flushed with PBS, and the heart, including the thoracic aorta, was removed. The aortic root was then separated from the aorta and embedded in Optimal Cutting Temperature (OCT) gel before storage at −80°C. Serial cross-sections beginning at the aortic valves were collected and measurements of lesion size were performed at the same point within the aortic root for each animal. Six serial sections per mouse were analyzed. In addition, Sudan IV staining of the thoracic aorta (n=5 per group) was used to assess the total surface area occupied by atherosclerosis.

**Quantification of Vascular Calcification**

Calcification of the aortic valves and atherosclerotic lesions of the aortic root was detected by von Kossa staining of 7-μm cryosections as previously described. Briefly, the cryosections were placed in 5% silver nitrate solution for 30 minutes in the absence of light. The sections were then treated with film developer (Ilford Ilfossol S) for 5 minutes and finally fixed in 5% sodium-thiolsulphate. Calcification was visualized as distinct black deposits of calcium using an epifluorescent microscope (Zeiss Axioscope 2, Carl Zeiss) coupled to an IBM computer (Hewlett Packard). All images were captured using a 3CCD video camera module and analyzed using the Northern Eclipse imaging software system (Empix Imaging Inc). Measurements of calcified area were measured by measuring calcification within the atherosclerotic lesions themselves and are expressed as a percentage of total lesion size. Similarly, measurements of valvular calcification were made by quantitating the amount of calcification tracking along the valvular surface and are expressed as a percentage of valvular surface area. All measurements of calcification and either lesion size or valvular surface area were made on the same sections.

**Alkaline Phosphatase Activity**

Measurements of ALP activity were used to quantify osteoblast differentiation in primary cultures of bovine aortic smooth muscle cells (BASMCs). Briefly, BASMCs were obtained from explant cultures as described previously. Then they were seeded into 24 well plates, at a concentration of 5×10^4 cells per well, and cultured for up to 6 days in DMEM containing 10% FBS and increasing concentrations of leptin. In some experiments, the cells were also cultured in the presence of the MEK inhibitor, PD98059, before treatment with leptin. At various times thereafter, the cells were harvested, lysed with 1% Triton X-100, and ALP activity assessed at 405 nm using a p-nitrophenol phosphate substrate kit (Sigma Chemical Co.). ALP values (U/mg) were normalized to protein using the Bio-Rad DC protein assay (Bio-Rad, Hercules).

Alkaline phosphatase staining of the aortic root was also used to identify osteoblast-like cells present in the atherosclerotic lesions of leptin-treated and nontreated mice. Briefly, slides were fixed in citrate buffered acetone for 30 seconds and then rinsed in deionized water. The slides were subsequently incubated in an alkaline-dye mixture (consisting of Fast Blue RR Salt and Naphth AS-MX Phosphate Alkaline Solution) at room temperature for 30 minutes. The percentage of total lesion area staining positive for alkaline phosphatase was then quantified using an epifluorescent microscope as described above.

**Preparation of Cell-Free Lysates**

Vascular smooth muscle cells were cultured to 70% confluence in DMEM containing 10% FBS before being stimulated with 2 μg/mL leptin. At various times thereafter, cell-free lysates were prepared and run on SDS-PAGE as described previously. Immunoblots were then performed using either an anti-Erk 1/2 antibody or an anti–P-Erk antibody and a horseradish peroxidase-conjugated secondary antibody.

**Osteocalcin and Osteopontin Immunofluorescence**

To confirm the presence of osteoblast-differentiation markers within the aortic root, osteocalcin goat antimouse polyclonal IgG and osteopontin goat antimouse polyclonal IgG primary antibodies were used. Briefly, cryosections were fixed in ice-cold acetone, washed in PBS, and blocked with 5% normal donkey serum and 2% BSA for 1 hour. Sections were incubated in primary antibody (4 μg/mL) overnight at 4°C before being incubated with AlexaFluor 594 at a 1:200 dilution (2 mg/mL, Invitrogen) for 1 hour. The sections were then visualized under oil immersion using the Zeiss Axioscope 2 (Carl Zeiss).

**Preparation of DIG-Labeled Riboprobes and In Situ Hybridization**

Total RNA was extracted from primary murine calvaria cells using an RNasy RNA mini kit (Qiagen) before being transcribed into single-stranded cDNA. Double-stranded cDNA was generated by polymerase chain reaction (PCR) using specific sense and antisense primers for osteopontin and osteocalcin that were designed to include the T7 RNA polymerase binding sequence on their 5′ ends. PCR products were run on a 1% agarose gel, and the positively identified DNA fragments were excised for subsequent purification using a QiAquick Gel Extraction Kit (Qiagen). DIG-labeling of the sense and antisense probes was performed using a DIG-RNA Labeling Kit and T7 RNA polymerase (Roche Diagnostics). The DIG-labeled sense and antisense riboprobes (50 ng/mL) were then used to perform in situ hybridizations on proteinase K–treated cryosections as described previously.

**Statistical Analysis**

Analysis of variance was used to compare the results in the experimental group with those in the control. A significance of differences was determined using unpaired Student t tests with a Bonferroni correction for multiple comparisons. All data are expressed as a mean±standard error (SEM).

**Results**

**Effect of Leptin on Body Weight and Plasma Lipid Levels**

Elevated leptin levels have been reported to cause weight loss and to increase lesion size in apoE-deficient mice. We therefore measured body weight and lipid levels in leptin-treated and nontreated apoE-deficient mice after a 2-month period. As seen in the Table, leptin-treated mice weighed...
significantly \( P<0.05 \) less than did age-matched vehicle control mice (19.5±0.3 g versus 20.7±0.5 g). In contrast, whereas plasma triglyceride and cholesterol levels were slightly higher in leptin-treated animals, this difference was not statistically significant when compared to age-matched vehicle control mice (1.59±0.07 mmol/L versus 1.47±0.12 mmol/L; \( P>0.05 \)) and (122±6.4 mmol/L versus 106±10.7 mmol/L; \( P>0.05 \), respectively; Table). Similarly, while blood glucose levels also tended to be higher in leptin-treated mice (10.84±0.84 mmol/L versus 9.31±0.57 mmol/L), this difference too was not statistically significant \( (P>0.05); \) Table). However, when fasting plasma insulin levels were measured, insulin levels were found to be significantly lower in the leptin-treated group \( (0.33±0.02\) versus 0.89±0.25; \( P<0.02 \)).

The Effect of Leptin on the Calcification of Atherosclerotic Lesions

Although several studies have suggested that leptin promotes atherosclerosis in apoE-deficient mice, to date none have examined the effect of leptin on vascular calcification in vivo. We therefore examined the effect of leptin on both lesion size and calcification using apoE-deficient mice. As can be seen in Figure 1A, lesion size within the aortic root of leptin-treated and nontreated animals did not differ significantly \( (9.5±1.5\times10^4 \mu m^2 \text{ versus } 10.6±2.0\times10^4 \mu m^2, \text{ respectively; } P>0.05) \). Similarly, no significant difference \( (P>0.2) \) in the percentage of surface area covered by lesion was found when the thoracic aorta of leptin-treated and nontreated animals was examined after lipid staining with Sudan IV (Figure 1B).

Next, to determine the effect of leptin on vascular calcification, we stained the aortic root of leptin-treated and nontreated apoE-deficient mice with von Kossa (Figure 2A versus 2B). As can be seen in Figure 2B, the lesions found within the aortic root of leptin-treated animals demonstrated both large and small punctate areas of calcification which stained black. As compared to nontreated animals, the lesions found within leptin-treated animals demonstrated significantly \( (P<0.001) \) more calcification (Figure 2C). Thus, those animals which received leptin demonstrated an 8.3±2.0-fold increase in the proportion of lesion area that was calcified as compared to nontreated animals \( (9.02±2.18\% \text{ versus } 1.09±0.20\%; \text{ } P<0.001; \text{ Figure 2C}). \)

The Effect of Leptin on Valvular Calcification

In addition to promoting the calcification of atherosclerotic lesions, leptin was also found to promote the calcification of the aortic valves. Thus, black punctate deposits denoting calcification were readily observable tracking along the length of the aortic valves in those animals treated with leptin (Figure 3B). Little or no staining was observed in those sections that were either obtained from nonleptin treated animals (Figure 3A) or left unstained (Figure 3C and 3D). When quantified, leptin-treated animals showed a 2.5±0.6-fold increase in valvular calcification as compared to nontreated animals \( (5.71±1.33\% \text{ versus } 2.29±0.64\%; \text{ } P<0.05)). \)

Effect of Leptin on Osteoblast Differentiation in Primary Cultures of Bovine Aortic Smooth Muscle Cells

Because leptin was found to promote the calcification of atherosclerotic lesions, we next decided to verify an earlier report\(^2\) of leptin inducing the osteogenic differentiation of vascular smooth muscle cells. Primary BASMCs were cultured in the presence or absence of leptin for increasing periods of time and the effect on BASMC differentiation determined by quantifying the amount of ALP activity in our vascular smooth muscle cell cultures. As seen in Figure 4A, ALP activity was significantly \( (P<0.01) \) and dose-dependently increased when BASMCs were cultured in the presence of increasing concentrations of leptin. Maximum

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**Table. The Effects of Leptin on Body Weight and Plasma Biochemistry**

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Control</th>
<th>Leptin-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>20.7±0.5</td>
<td>19.5±0.3*</td>
</tr>
<tr>
<td>Blood glucose, mmol/L</td>
<td>9.31±0.57</td>
<td>10.84±0.84</td>
</tr>
<tr>
<td>Plasma triglycerides, mmol/L</td>
<td>1.47±0.12</td>
<td>1.59±0.07</td>
</tr>
<tr>
<td>Plasma cholesterol, mmol/L</td>
<td>106±10.7</td>
<td>122±6.4</td>
</tr>
<tr>
<td>Plasma insulin, ng/ml</td>
<td>0.89±0.25</td>
<td>0.33±0.02*</td>
</tr>
</tbody>
</table>

Data are expressed as a mean±SEM. \(*P<0.05\) when compared to vehicle control.
ALP activity was seen when the BASMCs were cultured for 6 days in the presence of 2 μg/mL leptin (434±46 U/mg versus 151±12 U/mg; P<0.01).

In an effort to determine how leptin was acting to increase osteoblast differentiation in primary BASMC cultures, we next decided to examine the effect of leptin on extracellular-signal-related kinase (Erk) activation. Primary BASMCs were treated with leptin for increasing periods of time before lysing the cells and immunoblotting for either Erk1&2 or p-Erk1&2. As seen in Figure 4B, leptin treatment of BASMCs significantly increased Erk1 & 2 phosphorylation in a time-dependent manner (4.5-fold, P<0.05). Maximal stimulation of Erk was obtained when BASMCs were treated for 20 minutes with 2 μg/mL leptin and was sustained for at least 2 hours before returning to control levels (data not shown).

Finally, to determine whether Erk 1 & 2 activation by leptin was responsible for the ability of leptin to induce osteoblast differentiation in primary cultures of BASMCs, we treated BASMCs with leptin and increasing concentrations of the MEK inhibitor, PD098059 (C). The effect on either alkaline phosphatase activity (A and C) or Erk 1 & 2 activation (B) was then determined as described in the Materials and Methods section. Data are represented as a mean±SEM.

Figure 4. Effect of leptin on osteoblast differentiation in primary cultures of BASMCs. Primary BASMCs were cultured in the absence or presence of leptin (A and B) or leptin and the MEK inhibitor, PD098059 (C). The effect on either alkaline phosphatase activity (A and C) or Erk 1 & 2 activation (B) was then determined as described in the Materials and Methods section. Data are represented as a mean±SEM.
PD098059, the ability of leptin to induce ALP activity was significantly diminished.

**Effect of Leptin on Markers of Osteoblast Differentiation In Vivo**

Because we were able to confirm the ability of leptin to induce osteoblast differentiation in vitro, we next decided to look for evidence of osteoblast-like cells within the calcified lesions of animals treated with leptin. As seen in Figure 5A, areas staining positive for alkaline phosphatase (ALP) were observed within the atherosclerotic lesions and to a lesser extent the medial layer of leptin-treated animals. When quantified, the percent total lesion area demonstrating ALP positive staining was 5.4±2.1-fold \( (P<0.05) \) greater in leptin-treated mice as compared to nontreated age-matched control mice (1.72±0.69% versus 0.32±0.22%, respectively). Only half of the animals from the vehicle control group stained positive for ALP, whereas 6 of the 7 animals showed ALP staining in the leptin-treated group (data not shown).

To confirm our ALP findings, we next immunostained the lesions of leptin-treated and nontreated animals for several other osteoblast-specific markers. As seen in Figure 5B, the atherosclerotic lesions of our leptin-treated mice stained more intensely for the osteoblast-specific markers osteocalcin and osteopontin than did the lesions of nontreated animals. In addition, the expression of both osteocalcin and osteopontin was detected by in situ hybridization within the atherosclerotic lesions of leptin-treated animals (Figure 5C) and to a lesser extent in nontreated animals.

**Discussion**

By using a well-defined animal model of atherosclerosis, we have shown that the adipocytokine leptin promotes the calcification of atherosclerotic lesions in vivo. In addition, by measuring the expression of osteoblast-specific markers within the lesions of leptin-treated mice, we were able to demonstrate that this increase in vascular calcification is associated with an increase in osteoblast number. To our knowledge, this is the first report of leptin increasing vascular calcification in an in vivo animal model.

Several working hypotheses have been advanced in an attempt to explain how vascular calcification occurs. These include the presence of osteoblasts (bone-forming cells) within the vessel wall,\(^{30–33}\) the loss of specific calcification inhibitors such as matrix Gla protein,\(^{34–36}\) and finally the nonspecific entrapment of calcium.\(^{37,38}\) Based on the findings of the current study, however, we believe that vascular calcification results from the localization of osteoblast-like cells within the vessel wall. Thus, in the current study we were able to demonstrate that leptin not only promotes vascular calcification but that the increase in vascular calcification is associated with an increase in osteoblast-specific markers within the vessel wall. Indeed several investigators have also localized osteoblast-specific markers to the calcified atherosclerotic lesions of human vessels.\(^{30–33}\)

How leptin increases the expression of osteoblast-specific markers within the vessel wall is unknown. However, in the current study, we demonstrate that leptin can induce osteoblast differentiation in primary cultures of vascular smooth muscle cells (Figure 4A). In addition, we demonstrate that...
this effect is Erk1 & 2 dependent (Figure 4B and 4C). Thus, it is possible that leptin is promoting osteoblast differentiation within the atherosclerotic lesions of our apoE-deficient mice and that this is occurring in an Erk 1 & 2–dependent manner. Alternatively, leptin has been shown to increase true osteoblast differentiation within the bone marrow space, and thus it is possible that osteoblasts can, in low numbers, migrate within the circulation and thereby localize within the vessel wall at sites of atherosclerosis.

In the current study, leptin was found to not only promote the calcification of atherosclerotic lesions but also valvular calcification within the aortic root. Whether the process of vascular calcification at these 2 distinct sites occurs by similar mechanisms is not known. However, we could not unequivocally observe osteoblast-specific markers lining the valve surface. Interestingly, a number of studies have suggested an association between valvular aortic stenosis and valve surface. Interestingly, a number of studies have suggested an association between valvular aortic stenosis and high circulating levels of leptin. Although postinflammatory scarring can account for aortic stenosis, by far the most common cause of aortic stenosis is senile calcification of the heart valve(s).

Leptin-treated mice were found to weigh slightly less than age-matched vehicle control mice (19.5 ± 0.3 versus 20.7 ± 0.5 g; P < 0.05). A one gram difference in body weight (19.5 ± 0.3 versus 20.7 ± 0.5 g) represents an approximate 5% decrease in overall weight. Whether such a minor drop in body weight affected any of the other outcomes that we measured is unknown. Leptin-treated mice did show improved insulin-sensitivity when compared to nontreated mice. However, whether this occurred in response to the decrease in body weight or because of some yet to be defined mechanism is unknown. Interestingly, neither glucose levels nor plasma lipid levels showed any improvement when the animals, which were maintained on a high-fat diet, were also treated with leptin.

Our findings failed to show an increase in lesion size or total surface area occupied by lesions after the administration of leptin to apoE-deficient mice (Figure 1). At first glance, these findings appear to be at odds with previous reports that show leptin having proatherogenic effects. Thus, prior studies have shown that leptin increases total lesion area within the thoracic aorta of leptin-treated animals. However, when we examined the thoracic aorta of leptin-treated and nontreated apoE-deficient mice, we found no difference in the surface area occupied by lesions (Figure 1B). Why our findings are at odds with previous studies is not clear. However, in previous studies only male mice were used, whereas in our study only female apoE-deficient mice were used. Whether this accounts for the observed discrepancy is not known.

In addition to examining the effect of leptin within the thoracic aorta, we also examined its effects on lesion size within the aortic root. Again no effect on lesion size was observed. In previous studies, the proatherogenic effects of leptin were only examined within the carotid and brachiocephalic arteries or the thoracic aorta and not within the aortic root. Thus, in addition to gender differences, it is possible that the proatherogenic effects of leptin are site-specific and that lesion size is not increased by leptin within the aortic root regardless of gender. In support of this conclusion, Bodary et al. did not find a significant increase in total lesion area when examining lesions within the carotid artery of leptin-treated mice, even though the same animals showed a significant increase in total lesion area within the brachiocephalic artery and thoracic aorta. When taken together, our findings suggest that the effect(s) of leptin on vascular calcification are independent of any proatherogenic effects.

Our findings are supported by several clinical trials that have shown a positive correlation between circulating levels of plasma leptin and coronary artery calcification (CAC). Thus in a cross-sectional study conducted by Reilly et al., a positive correlation between plasma leptin levels and CAC was found in 200 type 2 diabetic subjects, even after controlling for other traditional risk factors such as body mass index, waist circumference, and C-reactive protein levels. Similar findings were reported in a separate study by Qasim et al., who examined CAC in 860 asymptomatic nondiabetic individuals. Thus, Qasim et al. examined the association of several plasma cytokines with CAC and found that only leptin was a significant independent predictor.

In summary, by treating apoE-deficient mice with exogenous leptin, we found that leptin promotes the calcification of atherosclerotic lesions without increasing lesion size, and that this is associated with an increase in osteoblast-specific markers found within the calcified lesions of leptin-treated mice. In addition, leptin treatment also appears to increase valvular calcification by a yet to be determined mechanism. When taken together, these findings may help to explain why individuals with high leptin levels have a higher incidence of CAC.

Disclosures

None.

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