Leptin Induces Vascular Smooth Muscle Cell Hypertrophy through Angiotensin II- and Endothelin-1-Dependent Mechanisms and Mediates Stretch-Induced Hypertrophy

Asad Zeidan, Daniel M. Purdham, Venkatesh Rajapurohitam, Sabzali Javadov, Subrata Chakrabarti, and Morris Karmazyn

Department of Physiology and Pharmacology (A.Z., D.M.P., V.R., S.J., M.K.) and Pathology (S.C.), University of Western Ontario, London, Ontario, Canada

Received June 24, 2005; accepted September 1, 2005

ABSTRACT

Various cardiovascular pathologies are associated with vascular smooth muscle cell (VSMC) hypertrophy and elevated plasma leptin levels. We used the rat portal vein (RPV) cultured for three days to investigate the effect of mechanical stretch on autocrine secretion of leptin and the effect of exogenous leptin (3.1 nM) on VSMC. Stretching the RPV significantly up-regulated leptin production by greater than 100-fold and leptin receptor expression by up to 10-fold. In addition, stretch increased tissue weight by 23 ± 1.3 and 30 ± 1% (P < 0.05), respectively, in the absence or presence of leptin, although this was significantly attenuated by an antileptin antibody (166 ng/ml). Unstretched RPV weight decreased by 7.5 ± 1.8% in the absence of leptin, whereas in the presence of leptin, weight increased by 6.5 ± 1.8% (P < 0.05). VSMC size and [3H]leucine incorporation rates were significantly increased by leptin in stretched and unstretched tissues. Leptin-induced hypertrophy was associated with significant extracellular signal-regulated kinase (ERK1/2) activation as well as increased expression of angiotensinogen, the angiotensin type 1 receptor as well as preproendothelin-1, and the endothelin type A receptor, whereas ERK inhibition or inhibition of either the angiotensin II or endothelin-1 systems at both the synthesis and receptor levels blocked the hypertrophic response. The effects of leptin were also completely blocked by the cholesterol-chelating agent methyl-β-cyclohextrin. Therefore, our study demonstrates stretch-dependent leptin release and a direct hypertrophic effect of leptin on RPV, the latter likely dependent on intact cholesterol-rich membrane microdomains and locally produced paracrine factors.

Leptin is a 16-kDa protein monomer produced mainly from adipocytes (Zhang et al., 1994), although recent studies have demonstrated its production from other sources, including stomach (Sobhani et al., 2000), heart (Purdham et al., 2004), and placenta (Masuzaki et al., 1997). Leptin exerts its biological activity through binding to its receptors termed OBR (also referred to as LEPR and LR, although for reasons of clarity, only the OBR designation is used in this report) found mainly in the hypothalamus (Bjorbaek and Kahn, 2004), although OBR have also been recently identified in peripheral vascular tissue, such as endothelial cells (Lembo et al., 2000), aorta (Oda et al., 2001), and the heart (Purdham et al., 2004). One of the key functions of leptin is regulation of body weight and metabolism by suppressing appetite via central mechanisms. Recent evidence has shown that leptin modulates not only regulation of body adiposity but other physiological actions, including regulatory effects on vascular tone and blood pressure (Matsumura et al., 2000), promoting angiogenesis (Cao et al., 2001), vascular cell calcification (Parrami et al., 2001), impairment of arterial distensibility (Singh et al., 2002), and induction of vascular smooth muscle cell (VSMC) proliferation and migration (Oda et al., 2001). Leptin has been shown to exert a negative inotropic effect in isolated cardiomyocytes (Nickola et al., 2000) as well as to

ABBREVIATIONS: OBR, leptin obese receptor(s); RPV, rat portal vein; VSMC, vascular smooth muscle cells; ERK1/2, extracellular signal-regulated kinase 1 and 2; mβCD, methyl-β-cyclohextrin; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; eNOS, endothelial nitric-oxide synthase; AT1, angiotensin type 1 receptor; ACE, angiotensin-converting enzyme; ET-1, endothelin-1; ETα, endothelin type A receptor; ECE, endothelin-converting enzymes; BO123, cyclo(Asp-Pro-o-Val-Leu-o-Trp); PD 98059, 2-amino-3-methoxy-flavone; PPRD, phosphoramidon; MES, 4-morpholineethanesulfonic acid.
produce hypertrophy in cultured neonatal rat ventricular myocytes (Rajapurohitam et al., 2003; Xu et al., 2004), whereas others have shown an antihypertrophic effect of leptin infusion in mice (Barouch et al., 2003). The signaling mechanisms underlying the effects of leptin are poorly understood, although mitogen-activated protein kinase (Banks et al., 2000), Janus kinase/signal transducers and activators of transcription (Ghilardi and Skoda, 1997), and nitric oxide (Vecchione et al., 2002) have been implicated in mediating some of the biological actions of leptin.

To date, the direct effects of leptin on VSMC have not been extensively examined especially to determine potential hypertrophic effects. Leptin levels are elevated in cardiovascular disorders; therefore, we considered it of importance to investigate whether leptin exerts any direct effects on blood vessels in terms of regulation of its production by mechanical stretch and the effect of exogenous leptin on VSMC hypertrophy. We also carried out a multifaceted approach to determine potential mechanisms for these effects, first by determining the role of the endogenous angiotensin and endothelin systems in mediating the effects of leptin, in view of the well established vascular hypertrophic effects of these peptides. Second, we determined the role of cholesterol-rich membrane microdomains/caveolae in mediating these effects because caveolae, which are present in a large number of mammalian cell types including VSMC (Zeidan et al., 2003), act as centers for coordinating signaling events in different tissues.

**Materials and Methods**

**Preparation of Isolated RPV and Experimental Protocol.** Male Sprague-Dawley rats weighing 200 to 250 g (Charles River Canada, Montreal, PQ, Canada) were killed by decapitation, and RPVs were removed, cleaned of fat and connective tissue, and cut longitudinally in two halves. The average weights of the RPV were 1.5 ± 0.1 mg. Because the RPV has a pronounced longitudinal layer of smooth muscle, the veins were cultured longitudinally either unstretched or subjected to a 0.6-g load for three days as described previously (Zeidan et al., 2003), a load slightly above that which is optimal for force development. The RPVs were cultured in culture medium with 2% fetal calf serum, 10 nM Leptin, and 2×10^(-5) M; Sigma-Aldrich), or antileptin antibody (166 ng/ml; Alpha Diagnostic International, Inc., San Antonio, TX) when used was added 1 h before initiating stretch or leptin addition.

**Measurement of Protein Synthesis.** After 48 h in culture, RPV strips were cultured for another 24 h with [H]leucine in order to assess the rate of protein synthesis as described previously (Zeidan et al., 2003). Tissue RNA Isolation and Real-Time PCR. Denuded RPVs were cultured for 6, 15, and 24 h in the absence or presence of leptin. After culturing, tissues were homogenized in TRIzol (Invitrogen, Carlsbad, CA) and RNA was isolated as per manufacturer’s instructions. One milligram of total RNA was used to synthesize the first strand of cDNA using random hexamer primers (Invitrogen) and SuperScript II Rnase H-Reverse Transcriptase (Invitrogen) according to the manufacturer’s protocol. The cDNA was diluted 10-fold, and 1 μl of the diluted cDNA was used in a 20-μl PCR reaction. The housekeeping gene 18S rRNA was used to normalize expression data. PCR conditions and cycle number were optimized for each gene. Table 1 shows primers and annealing temperatures used for each gene amplified by real-time PCR. 18S underwent 35 amplification cycles, whereas the other six genes underwent 40 cycles. PCR and melting curve analyses were performed in 20-ml reaction volumes using SYBR Green JumpStart Taq ReadyMix DNA polymerase (Sigma-Aldrich), and fluorescence was measured with a DNA Engine Opticon 2 System (MJ Research, Watertown, MA). Melting curve analysis showed a single PCR product for each gene amplified.

**Histology and Image Analysis.** After culturing for three days, RPVs were equilibrated under preload for 1 h and then fixed for electron microscopy as described previously (Zeidan et al., 2003). Electron micrographs were generated for measurement of VSMC surface area. The samples analyzed were from three to four portal veins for each treatment. Twenty-five to forty cells were randomly selected from each group, and the surface area was determined using image analysis software (Mocha; SPSS Inc., Chicago, IL).

**TABLE 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Annealing Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>GTAACCCGCTGGAACCCCATTT</td>
<td>CCA TCCATCTGGAAGTCG</td>
<td>55</td>
</tr>
<tr>
<td>Leptin</td>
<td>GAGACCTCCTCAGTGGCTTG</td>
<td>CTTGAGGCTTAAAGGCTC</td>
<td>58</td>
</tr>
<tr>
<td>OBRa</td>
<td>TGATATCGCCAAACGAGAAA</td>
<td>AGTGTGCGCTCTTTTGGA</td>
<td>58</td>
</tr>
<tr>
<td>OBRb</td>
<td>TGACCACTCCAGATCCTACACA</td>
<td>CACCTTTTTACACCTGCTG</td>
<td>58</td>
</tr>
<tr>
<td>OBRc</td>
<td>AAGTGGCTTAAAATCCCTTCG</td>
<td>CAAACATTGACAGGCCAAG</td>
<td>58</td>
</tr>
<tr>
<td>Angio</td>
<td>TGAGTTTCGGTTGGGCAACA</td>
<td>GAGGAGGCGGGTTCTTTATC</td>
<td>59</td>
</tr>
<tr>
<td>AT1</td>
<td>CAAAGGAGGAGTTGGGCTG</td>
<td>AGA GTTTGATGTTGGTGCA</td>
<td>58</td>
</tr>
<tr>
<td>PrePro ET-A</td>
<td>GGCAAAACTTTACACAGACCA</td>
<td>CAAACTTFAAAACCGCACA</td>
<td>58</td>
</tr>
<tr>
<td>ET-A</td>
<td>GTCTTCTGCTTGGTGCTCA</td>
<td>GG ACTTTGACAGAGTTTCA</td>
<td>58</td>
</tr>
</tbody>
</table>

Inhibitor PD 98059 (10 μM; Sigma-Aldrich), the AT1 receptor antagonist losartan (1 μM; Sigma-Aldrich), the angiotensin-converting enzyme (ACE) inhibitor captopril (10 μM; Sigma-Aldrich), the ET_a receptor antagonist BQ123 (1 μM; Sigma-Aldrich), the endothelin-converting enzyme inhibitor phosphoramidon (PPRD, 10 μM; Sigma-Aldrich), or antileptin antibody (166 ng/ml; Alpha Diagnostic International, Inc., San Antonio, TX) when used was added 1 h before initiating stretch or leptin addition.

**Determination of ERK Activation.** The determination of ERK1/2 phosphorylation was performed as described previously (Zeidan et al., 2000). In brief, RPVs were preincubated unstretched in culture medium with 2% fetal calf serum for 1 h and then acutely stretched or unstretched with or without 3.1 nM leptin for different durations up to 60 min. The strips were homogenized, and the total protein content was determined. ERK1/2 phosphorylation was analyzed by Western blot using anti-ERK1/2 and anti-P-ERK1/2 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).
Extraction/Replenishment of Cholesterol. Cholesterol was extracted by incubating tissues for 1 h at 30°C in HEPES-buffered Krebs solution (Zeidan et al., 2003) containing 5 mM methyl-β-cyclodextrin (mβcd; Sigma-Aldrich). For cholesterol repletion, tissues were incubated for 3 h at 30°C with 5 mg/ml cholesterol balanced with mβcd (water-soluble cholesterol; Sigma-Aldrich). After extraction/replenishment, tissues were extensively washed with HEPES-buffered Krebs solution before proceeding with the experiment.

Tissue Fractionation by Sucrose Gradient Centrifugation. RPV tissues were pulverized in liquid N₂ and suspended in 1 ml of 500 mM sodium carbonate, pH 11, and then sonicated with three 20-s bursts. The homogenate was brought to 45% sucrose by the addition of 1 ml of 90% sucrose in 25 mM MES, 150 mM NaCl, pH 6.5 (MES-buffered saline) and loaded in an ultracentrifuge tube. A discontinuous sucrose gradient was layered on top of the sample by placing 4 ml of 35% sucrose prepared in MBS with 250 mM sodium carbonate then 4 ml of 5% sucrose. The gradient was centrifuged at 39,000 rpm in a SW41Ti rotor (Beckman Instruments) for 21 h at 4°C. From the top, 1-ml fractions were collected to yield a total of 10 fractions. Equal volumes of each fraction were loaded in SDS-polyacrylamide gel followed by immunoblotting with a caveolin-1 (Santa Cruz Biotechnology, Inc.) and an OBR antibody (OBR12-A; Alpha Diagnostic International, Inc.).

Statistics. Values are presented as means ± S.E. Data were analyzed using one-way ANOVA followed by a post hoc Student’s t test. P < 0.05 was considered to represent significant differences between groups.

Results

The Hypertrophic Effect of Exogenous Leptin on VSMC Growth and Protein Synthesis. To investigate the effects of exogenous leptin on RPV growth and protein synthesis, denuded RPV strips were cultured for three days under stretched or unstretched conditions, with or without 3.1 nM leptin, a concentration within the range of plasma concentrations found in obese individuals (Maffei et al., 1995). Western blotting confirmed the total absence of eNOS protein in denuded vessels (data not shown). As shown in Fig. 1A, leptin significantly increased the RPV wet weight in stretched vessels by approximately 30%. In unstretched veins, tissue weight decreased by 7.5% after three days in culture, a finding in agreement with previous observations, although the precise mechanism for the loss in weight has not been elucidated (Zeidan et al., 2000, 2003). However, in the presence of leptin, the reduction in tissue weight was abrogated and vessel weight increased by 6.5%.

Leptin also significantly increased protein synthesis in both stretched and unstretched RPV, as determined by [³H]leucine incorporation (Fig. 1B). We also determined the relationship between wet and dry RPV weights in control and hypertrophied strips. There were no significant differences in the dry weight/wet weight ratios in stretched and unstretched vessels cultured in the presence or absence of leptin (data not shown). The mechanism by which leptin induces cell growth remains unclear.

Fig. 1. The hypertrophic effect of exogenous leptin on VSMC. Relative change in wet weight (A) and [³H]leucine incorporation (B) in RPV cultured under stretched or unstretched, with or without leptin (3.1 nM) is shown. C, effect of leptin on relative change of RPV length. D, representative electron micrographs of RPVs cultured for 3 days under various conditions. E, effect of leptin on VSMC size, evaluated from electron micrograph and normalized to the cell size of unstretched RPV cultured without leptin. Results are expressed as means ± S.E.M. of 6 (A and B), 8 (C), or 25 to 40 experiments (E). * P < 0.05 versus controls (unstretched without leptin). Bar in Fig. D = 1 μm.
stretched with and without leptin (0.17 ± 0.01 in stretched RPV with or without leptin versus 0.16 ± 0.01 or 0.17 ± 0.01 unstretched with or without leptin, respectively), thus indicating that increased tissue weights were not due to increased water retention.

**Morphological Effects of Leptin on VSMC.** Figure 1, D and E, demonstrate electron micrographs of tissues after various treatments and quantitative assessment of cell surface area, respectively. The cell surface area of the treated RPV with leptin under unstretched condition increased 57% compared with the control group. Stretch alone increased cell size by 143%, whereas in the presence of leptin, cell surface area significantly increased by 205%. These effects occurred in the absence of any evidence of mitosis as determined from the electron micrographs. We further studied the effect of treatments on RPV length. The mean RPV length before treatment was 9.7 ± 0.9 mm. As shown in Fig. 1C, leptin significantly increased the RPV length in both stretched and unstretched vessels.

**Effect of Stretch on Leptin and Leptin Receptors Expression and Release of Leptin from Vascular Smooth Muscle.** We next determined whether RPVs express leptin or its receptors. As shown in Fig. 2A, the expression of both leptin and leptin receptors was detected in these vessels, although very low levels were identified in resting tissues. However, in stretched tissues, a marked up-regulation of both leptin and OBR was evident, which ranged from approximately a 4-fold increase for OBRa to a 28-fold increase in leptin expression.

To determine whether these vessels can produce leptin, we collected culture media and measured leptin using an immunoassay in which the medium was changed on a daily basis. Low daily levels of leptin (generally <20 pg/ml) were detected in the culture media (Fig. 2B, left panel) collected from unstretched RPVs daily for each of the 3 days. However, stretching produced a massive increase in leptin release into the culture medium, although values declined at the third day of incubation. Figure 2B (right panel) illustrates total leptin concentrations in unchanged medium at the end of 3 days of culture. In this study, leptin concentrations increased from 34.5 ± 7.2 pg/ml in unstretched vessels to 2695 ± 75 pg/ml in tissues subjected to 3 days of stretch.

To ascertain whether stretch-induced hypertrophy was mediated by endogenous leptin release, stretched RPVs were incubated with or without antileptin antibody (166 ng/ml) for 3 days. As shown in Fig. 2, C and D, treatment with an antileptin antibody significantly attenuated stretch-induced increased tissue weight and protein synthesis, suggesting that the hypertrophic of RPVs to stretch is mediated, at least in part, by endogenously synthesized leptin.

**Effect of Leptin on ERK1/2 Phosphorylation.** To gain insight into the mechanism underlying the hypertrophic effect of leptin (growth and protein synthesis), we examined the role of early ERK1/2 activation in modulating this phenomenon. For this study, vessels were either left unstretched or acutely stretched for 60 min, both groups in the absence or presence of leptin during this period. Acute stretch alone increased ERK1/2 phosphorylation, which persisted for 30
min, whereas in the presence of leptin, the degree of ERK1/2 phosphorylation was enhanced (Fig. 3A). In unstretched tissues, leptin-induced ERK1/2 activation occurred 5 min after leptin addition, although the effect was transient with values returning to control levels thereafter (Fig. 3B). Peak ERK1/2 activation occurred 5 min after initiating acute stretch, therefore subsequent studies to determine ERK1/2 activation under various experimental conditions were done in 5-min stretched vessels.

Inhibition of ERK1/2 Activation Attenuates Leptin-Induced Hypertrophy. To determine whether ERK1/2 is involved in leptin-induced growth, we studied the effects of prevention of ERK1/2 activation by the MEK inhibitor PD 98059 on leptin-induced RPV growth and protein synthesis. As shown in Fig. 3, C and D, PD 98059 completely inhibited leptin-induced growth and protein synthesis in both stretched and unstretched RPV. In addition, stretch-induced cell growth in the absence of leptin was abrogated by PD 98059, suggesting that ERK1/2 mediates the effect of both leptin and stretch-induced cell growth.

Leptin Up-regulates Angiotensin and Endothelin Systems. We next studied the potential role of the angiotensin and endothelin systems in mediating the hypertrophic effects of leptin, first by determining whether these systems are up-regulated by exogenous leptin. As shown in Fig. 4, A and B, leptin significantly up-regulated both angiotensinogen and AT1 receptor expression, which peaked 15 h after leptin addition. In the case of the AT1 receptor, the up-regulation was transitory and returned to near baseline values 24 h after leptin addition. Moreover, leptin increased expression of prepro-ET-1, the precursor for ET-1 synthesis, as well as the ETA receptor 15 and 24 h after leptin administration.

Role of Local Angiotensin II and Endothelin 1 Synthesis on Leptin-Induced Growth, Protein Synthesis, and ERK1/2 Activation in RPVs. The observation that leptin up-regulates the angiotensin system led us to further investigate the involvement of local angiotensin II synthesis on leptin-induced growth, protein synthesis, and ERK1/2 activation by determining the effect of the ACE inhibitor captopril (1 μM) in unstretched vessels as well as those subjected to 5-min stretch. As shown in Fig. 5, A and B, captopril reduced leptin-induced growth and protein synthesis under both stretched and unstretched conditions. More-
over, captopril also completely prevented ERK1/2 activation (Fig. 6A), an effect that was mimicked by the AT1 receptor antagonist losartan (Fig. 6B). Essentially identical results were obtained with respect to the ET system in that both the endothelin-converting enzyme (ECE) inhibitor PPRD, which inhibits the conversion of big ET-1 to ET-1 and the ET\textsubscript{A} receptor antagonist BQ123, blocked both the hypertrophy (Fig. 5, A and B) and ERK1/2 activation (Fig. 6C) produced by leptin.

**Anti-Leptin Antibody Inhibits Leptin-Induced ERK1/2 Activation.** To verify the involvement of autocrine release of leptin in stretch-induced ERK1/2 activation, RPV strips were incubated with or without an antileptin antibody for 2 h before applying stretch for 5 min. Although, as shown in Fig. 6D, the antileptin antibody significantly inhibited stretch-induced ERK1/2 activation, the effect was only partial in nature. ERK1/2 phosphorylation levels were still significantly higher from control, thereby suggesting involvement of other additional pathways in stretch-induced ERK1/2 activation.

**Possible Role of Caveolae in Leptin-Induced Hypertrophic Responses.** The final set of studies determined the potential role of cholesterol-rich membrane microdomains/caveolae in mediating the effect of leptin on vascular hypertrophy. To assess the role of lipid raft/caveolae, tissues were treated with the cholesterol-chelating agent m\textsubscript{β}CD based on the fact that the structure and function of caveolae are sensitive to the amount of cholesterol in the membrane. It has previously been shown that incubation of RPV with 5 mM m\textsubscript{β}CD for 1 h, as used in the present study, has no effect on the RPV contractility, indicating a lack of cytotoxicity (Zeidan et al., 2003). As shown in Fig. 7, m\textsubscript{β}CD completely abrogated the effect of leptin on all indices of hypertrophy in both unstretched and stretched vessels. The effects of leptin were restored by the concomitant addition of cholesterol to m\textsubscript{β}CD-treated tissues.

Western blotting after sucrose gradient fractionation showed that caveolin-1, the primary caveolae protein in vascular tissue, was identified only in the low-density cholesterol-rich fractions (fraction 5; Fig. 7D, top panel). Moreover,
OBR with approximate molecular masses of 80 and 130 kDa (short and long receptor isoforms, respectively) were detected in the same fraction (fraction 5; Fig. 7D, bottom panel), strongly suggesting colocalization of OBR within lipid raft/caveolae.

**Discussion**

Leptin is a polypeptide hormone originally thought to be produced exclusively by adipocytes (reviewed by Ahima and Osei, 2004). In recent years, however, it has become evident that leptin can be locally produced by different tissues, such as cardiomyocytes, stomach, placenta, and, recently, aortic smooth muscle (Masuzaki et al., 1997; Sobhani et al., 2000; Oda et al., 2001; Purdham et al., 2004). Leptin exerts its biological actions through binding to its receptors termed OBR, with six splice variants of the receptor currently having been identified that have been designated as OBRa to OBRf (reviewed by Ahima and Osei, 2004). The long form (OBRb) is critical for leptin activity, whereas the function of the OBRa (short isoform) and OBRe is still poorly understood.

There are a number of major findings presented in our study that, to our knowledge, have not been previously reported and that may suggest a potential role of leptin in pathology associated with hyperleptinemic conditions. First is the ability of leptin to produce hypertrophy of RPV. Although leptin has been found previously to be a hypertrophic (Rajapurohitam et al., 2003; Xu et al., 2004) and hyperplastic (Tajmir et al., 2004) factor in cultured cardiomyocytes, it seems in the present study that the increase in wet weight of RPVs was due to a hypertrophic rather than a hyperplastic effect of the peptide. This concept is supported by a number of observations, including an increase in smooth muscle surface area and our inability to identify mitosis in stretched or unstretched vessels irrespective of the presence of leptin.

The hypertrophic effect of leptin strongly seems to be dependent on endogenous angiotensin II and ET-1 production based on various findings. First, leptin potently up-regulated gene expression of angiotensinogen and prepro-ET1, the precursors of both angiotensin II and ET-1, respectively. In addition, leptin increased the expression of both the AT1 and ETA receptor that occurred as early as 15 h after starting treatment and that was sustained for the treatment period, with the exception of the AT1 receptor, in which case the expression peaked at 15 h and then declined to near baseline values. Further evidence for a role of both angiotensin II and ET-1 in mediating the effects of leptin stems from studies involving selective pharmacological targeting of these systems. Thus, by preventing angiotensin II or ET-1 synthesis with an ACE or ECE inhibitor, respectively, or by blocking the AT1 or ETA receptor, the hypertrophic effect of leptin could be completely abrogated. Surprisingly, blocking either the ET or angiotensin systems completely prevented leptin-induced hypertrophy. The reason for this is uncertain at present, but the finding suggests that these systems act in concert and that both are required for the manifestation of the hypertrophic effect of leptin in these vessels. The exact
nature of the interrelationship between angiotensin II and ET-1 systems with leptin needs to be fully elucidated, but as recently reviewed (Barton et al., 2003), it is pertinent to note that local activation of both systems occurs in cardiovascular tissues in obesity. A relationship between leptin and either angiotensin II or ET-1 has indeed been proposed previously, when compared with unstretched vessels. One possible explanation for reduced leptin synthesis with chronic incubation may reflect a negative feedback effect by elevated angiotensin II, because a 3-day stretch has been shown to produce a more than 10-fold increase in local angiotensin II production (Bardy et al., 1996), which is in agreement with the present findings of up-regulated angiotensinogen and AT1 expression (Bardy et al., 1996), which is in agreement with the present findings of up-regulated angiotensinogen and AT1 gene expression. Further supporting this notion is the previous observation, alluded to above, that chronic administration of high-dose angiotensin II in rats results in a significant reduction in plasma leptin levels through a mechanism unrelated to blood pressure changes (Cassis et al., 1998).

The ability of an antileptin antibody to significantly atten-
uate the hypertrophic response to stretch, albeit incompletely, suggests a role for endogenously produced leptin in contributing to the stretch response in an autocrine or paracrine manner. The inability of the leptin antibody to completely prevent the hypertrophic response probably suggests that other factors, such as the activation of other receptors, also contribute to stretch-induced hypertrophy in addition to leptin. Moreover, it should be noted that, although stretch produced a massive increase in leptin release, the concentrations of leptin were still relatively low (low nanogram/milliliter levels) and hence probably insufficient to fully stimulate OBR under these conditions. In studies with exogenous leptin, either on unstretched or stretched vessels, a concentration of 50 ng/ml (3.1 nM) was employed because, as noted previously, this represents a concentration well within that found in obese individuals (Maffei et al., 1995).

The final potentially important finding in our study with respect to understanding mechanisms is the apparent importance of caveolae or at least cholesterol-rich microdomains in the leptin-induced VSMC hypertrophy as demonstrated by the ability of mβcd to abrogate this response and reversal of this effect by an exogenous water-soluble cholesterol preparation. Although mβcd has been used extensively to implicate caveolae in cellular processes, this agent acts via cholesterol depletion, suggesting other possible mechanisms by which it could reduce the hypertrophic effect of leptin not due to caveolae per se. However, extraction of cholesterol in these vessels has been shown to produce structural reorganization of the smooth muscle plasma membrane, which was marked by disassembly of caveolae (Zeidan et al., 2003). Our present study also demonstrates that OBR colocalize with caveolin based on tissue fractionation data. Indeed, it has been shown that ET-1-induced ERK1/2 activation in these vessels was significantly reduced after caveolae extraction (Zeidan et al., 2000). Accordingly, this may reflect an important if not the primary basis for inhibition of leptin-induced hypertrophy by caveolae extraction. A summary of the postulated sequence of events that may underlie the hypertrophic effect of leptin is provided in Fig. 8.

**Limitations of Study.** It should be recognized that a potential limitation of the present study is the use of isolated vascular tissue, which may not necessarily represent the in vivo situation. We chose to use this approach in our initial study to abrogate any potential role of circulating leptin on the vascular responsiveness to exogenous peptide as well as to preclude the potential central effects of the peptide, which can affect vascular function via the sympathetic nervous system. Moreover, results obtained in a venous tissue may not reflect effects on other vascular beds. Although the results obtained using the RPV could be of direct relevance to portal hypertension, the preparation was used because it has been well characterized in a number of recent publications (Zeidan et al., 2000, 2003; Albinsson et al., 2004) and the effect of increased RPV pressure (in vivo) due to partial occlusion in RPV has been studied (Malmqvist and Arner 1988, 1990). Because of its myogenic vasomotion, the RPV has been used in the development of vasoactive drugs as an analog of small precapillary resistance vessels (Lunge, 1990). Moreover, the longitudinally oriented musculature in RPV make it an ideal blood vessel for investigating the effect

![Fig. 8. Proposed model of hypertrophic effect of leptin on vascular smooth muscle. In brief, in this scheme, OBR located within caveolae (and blocked by mβcd) are activated by stretch or leptin, which then results in activation of ERK1/2 and a subsequent activation of both the angiotensin and endothelin systems. The hypertrophic response would then occur because of angiotensin II or ET-1 acting on AT1 or ETA receptors, respectively, and which would be blocked by the AT1 antagonist losartan or the ET_A receptor antagonist BQ123. Similarly, by preventing angiotensin II (Ang II) or endothelin-1 synthesis, hypertrophy would also be abrogated by the ACE inhibitor captopril or the ECE inhibitor PPRD, respectively. Transcriptional changes leading to hypertrophy would result subsequent to ERK1/2 or angiotensin II and ET-1 stimulation.](https://jpet.aspetjournals.org/doi/10.1124/jpet.107.123453)
of mechanical stress on muscle hypertrophy by stretching it by weight loading rather than perfusion.

Possible Clinical Relevance. The relevance of our findings in terms of understanding human cardiovascular pathologies remains to be determined with further studies in this relatively new field. It is attractive to speculate that these findings may be important in terms of understanding the potential role of leptin and hypertension such as the role that occurs in obesity in vascular disease, but perhaps especially in hypertension, which is associated with significantly elevated plasma leptin levels, independently of obesity (Henrikson et al., 2000). Of relevance to the present study, both plasma angiotensinogen and plasma leptin concentrations were previously positively correlated in young men with a positive family history of hypertension, although leptin but not angiotensinogen levels were also related to blood pressure in both hypertension-prone and nonprone groups (Schorr et al., 1998). OBR expression and plasma leptin levels are also elevated in obese hypertensive rats (Hiraoka et al., 1997). Although the precise contribution of leptin to vascular pathology, such as hypertension, needs to be better defined, it is pertinent to point out that hypertension is associated with vascular hypertrophy (Folkow, 1982) and it is possible that circulating or locally produced leptin contributes to this response, especially under hypertensive conditions seen in obese hypertensive individuals.

References
Barouch LA, Berkowitz DE, Harrison RW, O’Donnell CP, and Hare JM (2003) Leptin induces direct vasodilatation through distinct endothelial mecha


Address correspondence to: Dr. Morris Karmazyn, Department of Physiology and Pharmacology, University of Western Ontario, London, Ontario N6A 5C1, Canada. E-mail: morris.karmazyn@fdm.uwo.ca