Leptin Stimulates Ischemia-Induced Retinal Neovascularization

Possible Role of Vascular Endothelial Growth Factor Expressed in Retinal Endothelial Cells

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Diabetic retinopathy is the leading cause of new blindness in adults in developed countries. Leptin, an adipocyte-derived hormone, stimulates endothelial proliferation and angiogenesis. This study was designed to elucidate the pathophysiologic role of leptin in the progression of retinal neovascularization. Using the retinopathy of prematurity model, a mouse model of ischemia-induced retinal neovascularization, we have demonstrated more pronounced retinal neovascularization in 17-day-old transgenic mice overexpressing leptin than in age-matched wild-type littermates. Ischemia-induced retinal neovascularization was markedly suppressed in 17-day-old leptin-deficient *ob/ob* mice. Western blot analysis revealed that a biologically active leptin receptor isoform is expressed in mouse retinal endothelial cells. Leptin receptor expression was also detected in primary cultures of porcine retinal endothelial cells, where it upregulated vascular endothelial growth factor (VEGF) mRNA expression. This effect was thought to be mediated at least partly through the activation of signal transducers and activators of transcription (STAT)3, because adenoviral transfection of the dominant-negative form of STAT3 abolished the leptin-induced upregulation of VEGF mRNA expression in retinal endothelial cells. This study provides evidence that leptin stimulates the ischemia-induced retinal neovasucularization possibly through the upregulation of endothelial VEGF, thereby suggesting that leptin antagonism may offer a novel therapeutic strategy to prevent or treat diabetic retinopathy. *Diabetes* 53:2443–2448, 2004

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GFP, green fluorescent protein; IL, interleukin; PREC, porcine retinal endothelial cell; ROP, retinopathy of prematurity; STAT, signal transducers and activators of transcription; VEGF, vascular endothelial growth factor.

Diabetic retinopathy is the leading cause of new blindness in adults in developed countries (1). One of the possible contributors to the development of diabetic retinopathy is hyperglycemia, which leads to increased vasopermeability, endothelial cell proliferation, and neovascularization (2–4). Not all patients with poor control of diabetes over long periods of time, however, develop retinopathy, suggesting the involvement of other mechanisms.

Obesity, defined as increased mass of adipose tissue, is often associated with lifestyle-related cardiovascular and metabolic diseases such as diabetes, hypertension, and hyperlipidemia, thereby conferring a higher risk of vascular diseases or atherosclerosis. However, the molecular basis for that association is poorly understood. The adipose tissue is an important endocrine organ that secretes many biologically active substances such as free fatty acids, adiponectin, and interleukin (IL)-6. They are collectively termed adipocytokines (5). Evidence has accumulated suggesting that adipocytokines play pathophysiologic roles in obesity-related complications. Leptin is such an adipocytokine, acting directly on the hypothalamus, thereby regulating food intake and energy expenditure (6). The leptin receptor (Ob-R) is a single transmembrane protein that belongs to the gp130 family of cytokine receptor superfamily. The leptin receptor has several alternatively spliced isoforms, one of which, a biologically active Ob-Rb isoform, is expressed not only in the hypothalamus but also in a variety of peripheral tissues, suggesting the direct action of leptin in the periphery. The peripheral actions of leptin include the activation of platelet aggregation (7), the modulation of immune function (8), and the stimulation of vascular endothelial cell proliferation and angiogenesis (9,10). Upon binding to Ob-Rb, leptin has been shown to activate signal transducers and activators of transcription (STAT).

Numerous studies have revealed that plasma leptin concentrations are significantly elevated in obese subjects in proportion to the degree of adiposity (11), suggesting the pathophysiologic role of leptin in obesity-related complications. Indeed, we have demonstrated blood pressure

elevation as a result of sympathetic activation in transgenic skinny mice overexpressing leptin and suggested that leptin may be involved in some form of obesity-related hypertension (12,13). A recent study has revealed that plasma leptin concentrations are elevated significantly in patients with proliferative diabetic retinopathy relative to those with nonproliferative retinopathy (14). Furthermore, vitreous leptin concentrations are higher in patients with proliferative diabetic retinopathy or retinal detachment (15). These observations suggest that leptin may play a role in the development of diabetic retinopathy. However, whether leptin is causally related to the progression of diabetic retinopathy is currently undefined.

In this study, we sought to elucidate the pathophysiologic role of leptin in retinal neovascularization. We have examined the effect of leptin on retinal neovascularization in a mouse model of retinopathy of prematurity (ROP), which has proven to be useful in defining the mechanisms and possible treatment of diabetic retinopathy (1). This study provides evidence that leptin stimulates the ischemia-induced retinal neovascularization possibly through the upregulation of endothelial vascular endothelial growth factor (VEGF) in retinal endothelial cells, thereby suggesting that leptin antagonism may offer a novel therapeutic strategy to treat diabetic retinopathy.

RESEARCH DESIGN AND METHODS

Generation of transgenic skinny mice overexpressing leptin on the C57BL/6 background was reported previously (12). The transgene expression was under the control of the liver-specific human serum amyloid P component promoter, which is active during neonatal period. Ten-week-old male transgenic skinny mice were mated with female wild-type mice so as to produce transgenic and nontransgenic newborn mice. Ten-week-old male and female heterozygous *ob/+* mice on the C57BL/6 background (Charles River Japan, Yokohama, Japan) were used to produce wild-type +/+, heterozygous *ob/+*, and homozygous *ob/ob* newborn mice. Ischemia-induced retinal neovascularization model was performed as described (1,16). Briefly, litters of 7-day-old (postnatal day 7 or P7) mice, along with their nursing mothers, were exposed to 75 ± 2% oxygen for 5 days and then returned to room air on P12. Mice of the same age maintained in room air served as controls. At P17, they were sacrificed under pentobarbital anesthesia (30 mg/kg). All experimental protocols were approved by the Kyoto University Graduate School of Medicine Committee on Animal Research.

Analysis of neovascularization. Mice were perfused from the left ventricle with high-molecular-weight (2,000 kDa) fluorescein-conjugated dextran (Sigma, St. Louis, MO) in PBS solution (1,16). Eyes were enucleated and fixed in 4% paraformaldehyde. The retinas were dissected, radially cut, and viewed by fluorescence microscopy.

Retinal neovascularization was quantified as follows. Eyes were enucleated, fixed in 4% paraformaldehyde, and embedded in paraffin. Fifty serial sections (6 μm) starting at the optic nerve head were placed on microscope slides. After staining with periodic acid-Schiff, 10 intact sections of equal length, each 30 μm apart, were evaluated for a span of 300 μm. All retinal vascular cell nuclei anterior to the internal limiting membrane were counted in each section by a fully masked protocol. The mean of all 10 counted sections yielded average neovascular cell nuclei per 6-μm section per eye (1,16).

Cell cultures. Primary cultures of porcine retinal endothelial cells (PRECs) were isolated by homogenization and a series of filtration steps as described (17,18). After the cells reached confluence, cells from passages 3 to 8 were used in this study.

For hypoxic experiments, PRECs were exposed to 1% oxygen using an advanced computer-controlled infrared water-jacketed multigas incubator (model BL-M10; Jujikagaku, Tokyo, Japan). Cells were stimulated with 100 ng/ml leptin or vehicle and incubated with 1% oxygen for 12 h. Cells maintained under these conditions for periods >12 h showed no morphologic changes by light microscopy and could subsequently be passaged. Cells incubated under standard normoxic conditions (95% air and 5% CO_2) from the same batch and passage were used as controls (19,20).

Western blot analysis. Phosphorylation of STAT3 was measured by Western blotting as previously described (16). PRECs were lased on ice in solution that contained Laemmli buffer (50 mmol/l Tris [pH 7.5], 2% SDS, and 10% glycerol) containing phosphatase inhibitors (1 mmol/l NaF and 0.5 mmol/l Na3VO4) and protease inhibitors (10 mmol/l sodium pyrophosphate, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 mmol/l phenylmethylsulfonyl fluoride). Total cell lysate (30 μg) was subjected to SDS-PAGE under reducing conditions, and proteins were transferred to nitrocellulose membrane (Bio-Rad, Richmond, CA). After the membranes were incubated with primary antibodies at 4°C overnight, immunoblots were developed with a horseradish peroxidase–conjugated secondary antibody (Amersham, Arlington Heights, IL) and analyzed by enhanced chemiluminescence using a commercially available kit (ECL Plus; Amersham). The primary antibodies used in this study were anti-leptin receptor Ob-Rb (Santa Cruz Biotechnology, Santa Cruz, CA), anti–phospho-STAT3, and anti-STAT3 antibodies (Upstate Biotechnology, Lake Placid, NY).

Immunohistochemistry. For immunofluorescence study of Ob-Rb, 6-μm thick paraffin-embedded sections were incubated with primary antibody. After incubation with a fluorescein-labeled secondary antibody, the sections were incubated with Alexa-fluor 594–conjugated lectin (Molecular Probes, Eugene, OR). They were viewed and photographed by confocal microscopy (Carl Zeiss, Munich, Germany). For immunostaining, a standard indirect immunoperoxidase protocol using a commercially available kit (Elite ABC; Vector Laboratories, Burlingame, CA) was performed with diaminobenzidine tetrahydrochloride (DAB; Dako) as substrate. For negative control, the primary antibody preincubated with the immunizing peptide (Santa Cruz Biotechnology) was used. Hematoxylin and eosin staining was also performed on adjacent sections to evaluate general pathologic changes.

Real-time quantitative RT-PCR. Total RNA was extracted from P14 mice retinas by the acid guanidinium-phenol-chloroform method and treated with DNase. cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Amersham). The VEGF probe (5′-Fam-TGCCAAGTGGTCCC-3′) with a minor groove binder (21), forward primer (5′-ACCCTGGCTTTACTGCTGTACC-3′), and reverse primer (5′-CAAGGCCCACAGGGATTTT-3′) were designed using the ABI PRISM Primer Express 2.0 (Applied Biosystems, Foster City, CA). Real-time PCR was done using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). Each PCR amplification was performed in triplicate under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 s at 95°C and 1 min at 60°C).

Northern blot analysis. Northern blot analysis was performed using the ^{32}P-labeled mouse VEGF cDNA probe (19). As an internal control, the membranes were rehybridized with the ^{32}P-labeled 36B4 cDNA control probe (19). Autoradiography was performed using BAS-2500 system (Fuji Photo Film, Tokyo, Japan).

Adenoviral transfection. PRECs were transfected with adenoviral vectors expressing a dominant-negative form of STAT3 or constitutively activated STAT3 and incubated for 12 h (22). After removal of viral suspension, PRECs were serum starved for 8 h and stimulated with leptin. Adenoviral vector for green fluorescent protein (GFP) (Clontech Laboratories, Palo Alto, CA) was used as a control.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed by use of ANOVA followed by Scheffe's test. $P < 0.05$ was considered statistically significant.

RESULTS

Effects of leptin overexpression or its deficiency on retinal neovascularization. To investigate the pathophysiologic role of leptin in the progression of ischemia-induced retinal neovascularization, we examined the flat-mounted whole retinas from control wild-type mice, leptin-deficient *ob/ob* mice, and transgenic mice overexpressing leptin (12). Without oxygen treatment, there was no significant difference in retinal neovascularization area among genotypes (control wild-type mice [Fig. 1*A*] and *ob/ob* and transgenic skinny mice [not shown]). Significant areas of neovascularization were detected in flat-mounted whole retinas from oxygen-treated control wild-type mice (Fig. 1*B*). Neovascularization was markedly suppressed in retinas from oxygen-treated leptin-deficient *ob/ob* mice relative to oxygen-treated wild-type littermates (Fig. 1*C*). By contrast, extensive neovascularization was observed in retinas from oxygen-treated transgenic mice relative to oxygen-treated wild-type littermates (Fig. 1*D*).

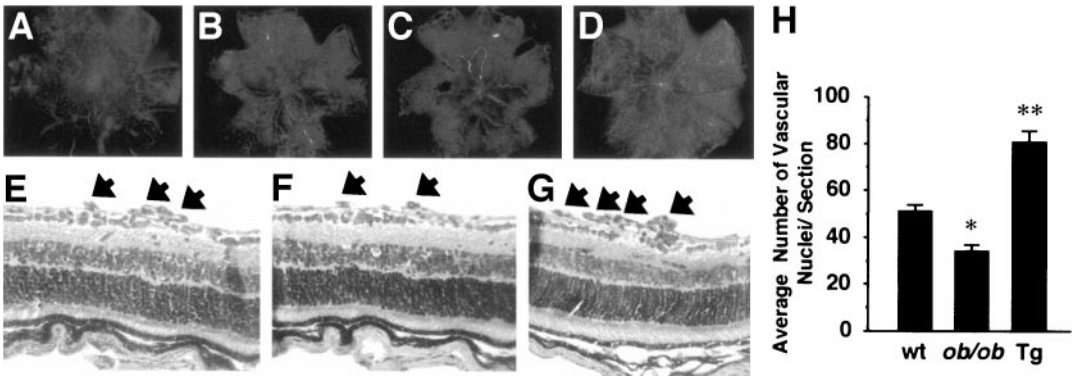

FIG. 1. Retinal vascular pattern by fluorescein-perfused retinal flat mount of wild-type mice (*A* and *B*), *ob/ob* mice (*C*), and transgenic mice overexpressing leptin (*D*) with (*B–D*) or without (*A*) oxygen treatment. Five days after oxygen treatment (P17), fluorescence perfusion showed that the vasculature is substantially increased in transgenic mice and reduced in *ob/ob* mice compared with wild-type mice. Cross-sectional analysis of retina from wild-type mice (*E*), *ob/ob* mice (*F*), and transgenic mice (*G*). Arrows indicate neovascular tufts. Periodic-acid Schiff stain, ×400. *H*: Quantitative analysis of retinal neovascularization by counting the neovascular cell nuclei. **P* < 0.05, ***P* < 0.01 vs. wild-type mice; *n* = 9–10. Tg, transgenic mice; wt, wild-type mice.

A histological examination of ocular cross sections revealed negligible numbers of nuclei protruding above the inner lining membrane in retinas from control mice, *ob/ob* mice, and transgenic mice maintained in normoxia (data not shown). The wild-type mice exposed to relative hypoxia showed 51.0 ± 2.4 neovascular nuclei/section, whereas the number of neovascular nuclei in retinas from *ob/ob* mice was significantly reduced to 34.1 ± 3.2 ($P < 0.05$) (Fig. 1*E*, *F*, and *H*). By contrast, neovascular nuclei were significantly increased in retinas from transgenic mice overexpressing leptin (80.2 ± 4.8 nuclei/section, $P < 0.01$) (Fig. 1*G*).

Expression of leptin receptor in retinal endothelial cells. To assess the site of action of leptin in retinal vessels, we examined the localization of leptin receptor (Ob-Rb) expression in mouse retina. Western blot analysis revealed that Ob-Rb protein is expressed abundantly in the porcine hypothalamic tissue (Fig. 2*A*). In this study, no significant amount of Ob-Rb protein is detected in the cerebral tissue. Ob-Rb protein is also abundant in retinal endothelial cells, whereas no obvious band is found in retinal glial cells (Fig. 2*A*). Immunohistochemical analysis revealed that some endothelial cells (red, lectin positive) are positive for Ob-Rb (green) in the neovascular tufts of wild-type mice treated with oxygen (Fig. 2*B*). There were few double positive cells in retinas from wild-type mice without the oxygen treatment (data not shown). Immunostaining of Ob-Rb was predominantly localized in vascular cells in ganglion cell layer and neovascular tufts from wild-type mice treated with oxygen, and no obvious staining was observed in other cell layers (Fig. 2*C–E*).

Induction of VEGF mRNA in the retina after the oxygen treatment. Since it is well-known that VEGF plays a critical role in retinal neovascularization (23,24), we investigated VEGF mRNA expression in the mouse retina (Fig. 3). Real-time quantitative RT-PCR analysis revealed that the induction of VEGF mRNA expression after the oxygen treatment is significantly suppressed in *ob/ob* mice relative to wild-type mice (−73%, $P < 0.05$, $n = 6$). By contrast, transgenic mice overexpressing leptin showed more marked induction of VEGF mRNA

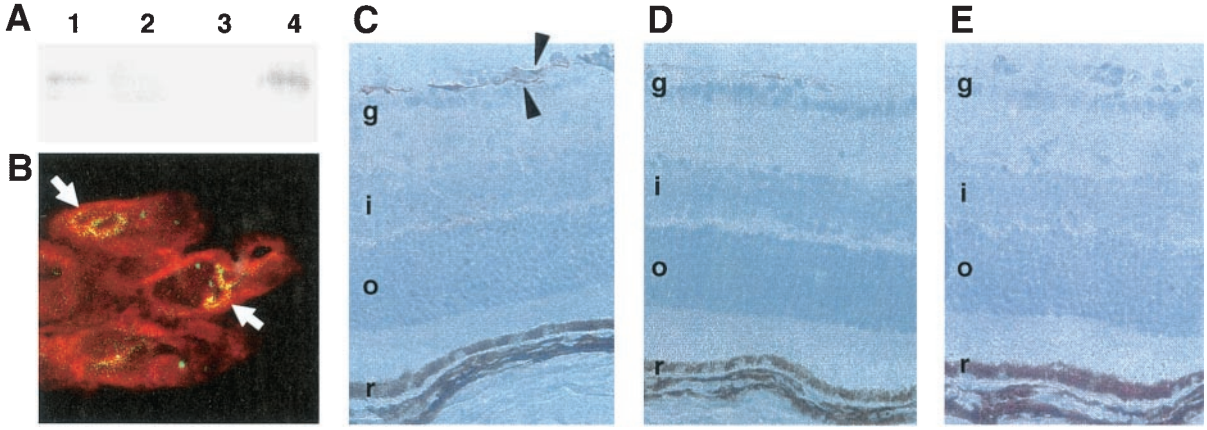

FIG. 2. Ob-Rb expression in retinas. *A*: Western blot analysis of Ob-Rb in cultured cells from the porcine retina and cerebral tissues. *Lane 1*, cultured retinal endothelial cells; *lane 2*, cultured retinal glial cells; *lane 3*, cerebral cortex; *lane 4*, hypothalamus. Equal amounts of protein (30 μg/lane) were subjected to Western blotting. *B*: Double staining of Ob-Rb (green) and lectin (red, endothelial cell marker) in the retina. Arrows indicate colocalization of Ob-Rb and lectin in neovascular tufts of wild-type mice with oxygen treatment. *C*: Immunostaining of Ob-Rb in a cross section of the retina of wild-type mice with oxygen treatment. Arrowheads indicate immunostaining of Ob-Rb in neovascular tufts. *D*: Immunostaining of Ob-Rb in the retina of wild-type mice without oxygen treatment. *E*: Negative control; the primary antibody preincubated with the immunizing peptide was used. The ganglion cell layer (g), inner nuclear layer (i), outer nuclear layer (o), and retinal pigment epithelium (r) are indicated in *C–E*.

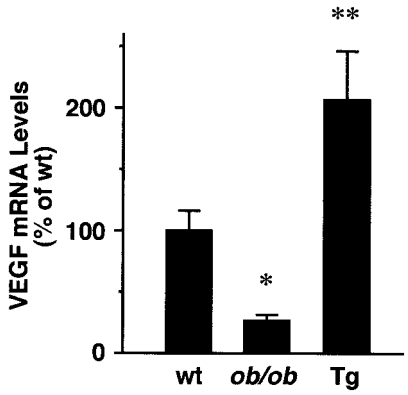

FIG. 3. Gene expression of VEGF in the retina from each strain after oxygen treatment. Real-time quantitative RT-PCR revealed that upregulation of VEGF mRNA was significantly attenuated in *ob/ob* mice compared with wild-type mice. In contrast, VEGF mRNA levels in leptin transgenic mice were much higher than those in wild-type mice. *$P < 0.05$, **$P < 0.01$ vs. wild-type mice; $n = 6$.

expression relative to wild-type littermates (107%, $P < 0.01$, $n = 6$).

Leptin-induced VEGF expression through STAT3 activation in retinal endothelial cells. To explore the molecular mechanisms underlying the differences of retinal neovascularization among genotypes, we examined the direct action of leptin in PRECs. Northern blot analysis showed that leptin dose-dependently upregulates VEGF mRNA expression in PRECs (Fig. 4*A*). Leptin at a dose of 1,000 ng/ml resulted in a threefold increase in VEGF mRNA expression relative to vehicle-treated groups. The maximal induction was observed 6 h after the leptin treatment (Fig. 4*B*). We also examined the effect of leptin on STAT3 phosphorylation. Leptin induced a transient tyrosine phosphorylation of STAT3, peaking at 15 min after stimulation (156%, $P < 0.05$, $n = 4$) (Fig. 4*C*). PRECs transfected with constitutively activated STAT3 showed a significant increase in VEGF mRNA expression relative to control adenovirus expressing GFP (122%, $P < 0.01$, $n = 6$) (Fig. 4*D*). Furthermore, transfection of dominant-negative STAT3 significantly inhibited the leptin-induced VEGF mRNA expression relative to control adenovirus expressing GFP (−60%, $P < 0.01$, $n = 6$) (Fig. 4*D*).

Effect of leptin on hypoxia-induced VEGF upregulation. Previous studies have shown that hypoxia is a strong inducer of VEGF gene expression and that relative hypoxia after the oxygen treatment plays a critical role in retinal neovascularization found in the hypoxia-induced ROP model. We therefore investigated the effect of leptin on the hypoxia-induced upregulation of VEGF mRNA expression in PRECs. Hypoxia increased VEGF mRNA expression by 89% in PRECs relative to those maintained under normoxic condition ($P < 0.05$, $n = 6$) (Fig. 5). Leptin significantly augmented the hypoxia-induced VEGF mRNA expression (170% of normoxic condition, $P < 0.05$, $n = 6$). These observations indicate that leptin has an additive effect on hypoxia-induced VEGF mRNA expression.

DISCUSSION

The ischemia-induced retinal neovascularization often results in catastrophic loss of vision in the final stage of various ocular diseases, including diabetic retinopathy, retinal vein occlusion, and ROP. It has been recognized that leptin promotes neovascularization and angiogenesis via direct vascular mechanisms (9,10). In this study, we have demonstrated for the first time that leptin stimulates the ischemia-induced retinal neovascularization in a mouse model of ROP (1). A recent study has revealed that plasma and vitreous leptin concentrations are correlated with the degree of diabetic retinopathy (14,15). These observations suggest that leptin may be involved in the development of diabetic retinopathy. Plasma leptin concentrations are elevated significantly in obese subjects in proportion to the severity of the disease (11), suggesting that obese subjects are resistant to the central antiobesity effect of leptin (termed leptin resistance). In this context, several recent studies have demonstrated that vascular remodeling and neointimal formation are markedly attenuated in leptin-deficient *ob/ob* mice or *db/db* mice with leptin receptor mutation (25,26). Therefore, hyperleptinemia may promote vascular injury in obese subjects. Given the good correlation between plasma leptin concentra-

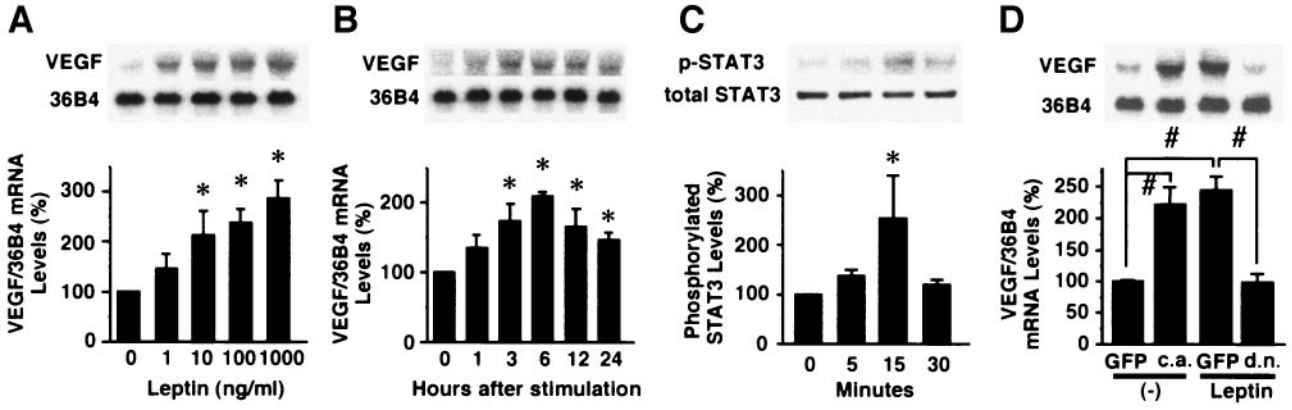

FIG. 4. Role of the leptin signaling in VEGF mRNA induction in cultured PRECs. *A*: Dose-dependent induction of VEGF mRNA by leptin treatment in PRECs. PRECs were treated with various concentrations of leptin (0, 1, 10, 100, and 1,000 ng/ml) for 6 h. *$P < 0.05$ vs. 0 ng/ml; $n = 6$. *B*: Time course of leptin-induced VEGF gene expression. PRECs were treated with 100 ng/ml leptin for the time indicated (0, 1, 3, 6, 12, and 24 h). *$P < 0.05$ vs. 0 h; $n = 6$. *C*: Phosphorylation of STAT3 by leptin treatment in PRECs. PRECs were treated with 100 ng/ml leptin for the time indicated (0, 5, 15, and 30 min). *$P < 0.05$ vs. 0 min; $n = 4$. *D*: Role of STAT3 in leptin-induced VEGF expression in PRECs. c.a., constitutively activated STAT3; d.n., dominant-negative STAT3. #$P < 0.01$; $n = 6$.

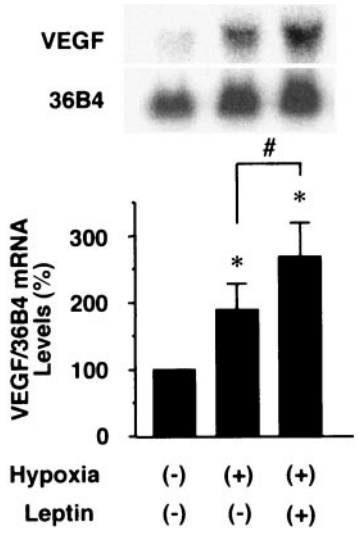

FIG. 5. Effects of leptin on hypoxia-induced VEGF mRNA expression in PRECs. Cells were stimulated with 100 ng/ml leptin or vehicle and incubated with 1% oxygen for 12 h. Leptin significantly enhanced hypoxia-induced VEGF mRNA expression. *$P < 0.05$ vs. basal level; #$P < 0.05$; $n = 6$.

tions and adiposity, the association between the degree of diabetic retinopathy and adiposity should be examined carefully. It is well recognized that hyperglycemia is one of the possible contributors to the development of diabetic retinopathy (2–4). In this regard, it is interesting to examine the degree of diabetic retinopathy in patients with lipoatrophic diabetes who have hyperglycemia but hypoleptinemia (27).

The molecular basis of the leptin-induced retinal neovascularization is currently unclear. In this study, leptin activated STAT3 in retinal endothelial cells, as revealed by increased STAT3 phosphorylation (Fig. 4*C*). Furthermore, the leptin-induced VEGF mRNA expression was abolished by adenoviral transfection of dominant-negative STAT3 (Fig. 4*D*). These observations, taken together, suggest that leptin increases VEGF mRNA expression in retinal endothelial cells by STAT3 activation. This notion is consistent with the presence of STAT3 binding sites in the 5′ flanking region of the human VEGF gene (28). Since VEGF plays a critical role in the proliferation of retinal endothelial cells (23,24), it is likely that leptin stimulates retinal neovascularization through the upregulation of endothelial VEGF and/or synergistically with VEGF (29).

Since glial cells are a major source of VEGF produced in the retina (30), it is important to examine whether glial cell–derived VEGF contributes to the leptin-induced retinal neovascularization. However, we have found that leptin cannot increase VEGF mRNA expression in cultured glial cells (unpublished data), which may be partly due to the fact that Ob-Rb protein is not expressed in retinal glial cells (Fig. 2*A*). We have reported major contribution of angiopoietin 2 (20) and neuropilin-1 (31), an isoform-specific receptor for $VEGF_{165}$ in ischemia-driven retinal neovascularization. Using mice with a vascular endothelial cell–specific knockout of insulin receptor, Kondo et al. (32) also showed that reduction in retinal neovascularization is accompanied by suppression of retinal expression of VEGF, endothelial nitric oxide synthase, and endothelin-1, suggesting the importance of such endothelial mediators in the development of retinal neovascularization. Whether leptin can stimulate endothelial production of such molecules in the retina must await further investigation. The above discussion supports the implication of endothelial cell–derived angiogenic factors such as VEGF in leptin-induced retinal neovascularization.

IL-6 is another adipocytokine in which expression is upregulated in the adipose tissue from obese animals and humans (33). It is capable of activating STAT3 similarly to leptin. Vitreous concentrations of IL-6 are higher in patients with active proliferative diabetic retinopathy than in those with inactive retinopathy (34). It has been demonstrated that IL-6 induces angiogenesis by the induction of VEGF (35). These findings suggest that IL-6 is also involved in the development of retinal neovascularization through the activation of VEGF. No apparent correlation has been reported between plasma IL-6 concentrations and the severity of retinal neovascular diseases. Given that leptin and IL-6 share STAT3 as an intracellular signaling molecule, it is tempting to speculate that IL-6 derived from the adipose tissue also plays a role in the development of retinal neovascularization.

In conclusion, this study represents the first demonstration that leptin plays a critical role in ischemia-induced retinal neovascularization. The angiogenic effect of leptin may be mediated at least in part through the upregulation of endothelial VEGF. Our data also suggest that leptin antagonism may offer a novel therapeutic strategy to prevent or treat diabetic retinopathy.

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