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Leptin stimulates type I collagen production in *db*/*db* mesangial cells: Glucose uptake and TGF-β type II receptor expression

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Leptin stimulates type I collagen production in *db*/*db* mesangial cells: Glucose uptake and TGF-β type II receptor expression.

Background. Serum leptin levels correlate with fat cell mass and are elevated in patients with massive obesity and type 2 diabetes mellitus, which are strong risk factors for the development of glomerulosclerosis. We have previously shown in cultured glomerular endothelial cells that leptin stimulates cellular proliferation and expression of the prosclerotic cytokine transforming growth factor-β1 (TGF-β1). Although the effect of leptin on the hypothalamus to regulate energy homeostasis is well known, the effect of leptin on the kidney, and specifically on the glomerular mesangial cell, is unclear.

Methods. The obese, diabetic *db*/*db* mouse, which lacks the functional full-length Ob-Rb leptin receptor, is a suitable model to assess the effects of hyperleptinemia on peripheral tissues that express other receptor isoforms. The effects of leptin on glucose uptake, the TGF-β system, and type I collagen production were evaluated in *db*/*db* mouse mesangial cells in culture. A phosphatidylinositol-3 kinase (PI-3K) inhibitor was used to assess the role of PI-3K in mediating the effects of leptin.

Results. A short form of the leptin receptor (Ob-Ra), but not Ob-Rb, was present by reverse transcription-polymerase chain reaction in the kidney and mesangial cells of both nondiabetic *db*/*m* and diabetic *db*/*db* mice. In *db*/*db* mesangial cells, leptin increased 2-deoxy-D-glucose (2DOG) uptake dose dependently and stimulated gene expression of TGF-β type II receptor (TβRII) and α1(I) collagen, but not TGF-β1. Protein production of type I collagen (enzyme-linked immunosorbent assay) was also increased by leptin. Both leptin-stimulated 2DOG uptake and type I collagen production were suppressed by a PI-3K inhibitor, LY294002. Mesangial cells pretreated with leptin exhibited increased responsiveness to exogenous TGF-β1, as evidenced by a greater production of type I collagen protein in leptin-pretreated cells exposed to low-dose TGF-β1 (0.5 ng/mL). The addition of both TGF-β1 (2 ng/mL) and leptin (100 ng/mL) increased type I collagen production more than addition of either TGF-β1 or leptin alone.

Conclusions. Leptin increases glucose uptake and type I collagen in *db*/*db* mesangial cells through a PI-3K–dependent pathway. We postulate that increased leptin levels may transmit a signal through the short-form leptin receptor to up-regulate TβRII and activate the intraglomerular TGF-β system, which may contribute to the glomerulosclerosis of obesity or type 2 diabetes.

Key words: glomerulus, glomerulosclerosis, obesity, diabetic nephropathy, progressive renal disease, adipocyte-derived hormone.

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Leptin, an adipocyte-derived hormone, interacts with the full-length leptin receptor [obese receptor b (Ob-Rb)] in the hypothalamus, thereby regulating appetite and energy expenditure [1]. Compared with these well-known neuronal actions, the extra-neuronal functions of leptin are less well characterized. Various truncated receptor variants (short-forms Ob-Ra, Ob-Rc, Ob-Rd, Ob-Re) have been described in peripheral tissues, including the kidney [2, 3], but the signaling potential of these variants and the role of leptin in the metabolic processes of peripheral tissues remain controversial. Recently, the direct effects of leptin have been demonstrated in cultured cells from various peripheral tissues in which Ob-Ra is predominantly expressed [4–8].

Differential modulation of glucose metabolism may explain the diverse actions of leptin on different peripheral tissues. Treatment with leptin in both wild-type mice and streptozotocin-diabetic rats increases glucose turnover and glucose uptake in peripheral tissues [9, 10]. Leptin treatment in the *ob*/*ob* mouse, which is leptin-deficient, decreases serum glucose and insulin levels, but leptin treatment in the *db*/*db* mouse, which is hyperleptinemic, does not significantly affect serum glucose or insulin levels [11, 12]. However, recent in vitro studies have demonstrated that leptin induces glucose uptake and glycogen synthesis through a phosphatidylinositol-3 kinase (PI-3K)–dependent pathway in C2C12 myotubes

[6, 13, 14]. Leptin also activates PI-3K in cultured hepatocytes through the short forms of the leptin receptor [8]. Therefore, leptin may affect glucose metabolism in different ways, depending on the levels of glucose and insulin, the type of tissue, and the expression of different leptin receptor isoforms.

Massively obese patients have high serum leptin levels [15] and tend to develop focal glomerulosclerosis [16]. Similarly, type 2 diabetic patients with hyperinsulinemia and an elevated body mass index exhibit high serum leptin levels [17] and are also at risk for developing glomerulosclerosis, especially in the mesangial space. Furthermore, the presence of microalbuminuria or macroalbuminuria predicts a significantly increased serum leptin level [18, 19]. These observations have led to the hypothesis that hyperleptinemia may contribute to the pathogenesis of renal fibrosis in these diseases.

A mutation in the full-length Ob-Rb leptin receptor in the *db*/*db* mouse leads to leptin resistance, high circulating leptin levels, obesity, and type 2 diabetes mellitus [20]. The *db*/*db* mouse also develops significant proteinuria and glomerulosclerosis similar to the course of diabetic nephropathy in humans [21]. Thus, this mouse model is suitable for assessing the effects of hyperleptinemia on peripheral tissues that express other receptor isoforms. Interestingly, our previous study in cultured glomerular endothelial cells demonstrated that leptin stimulates cellular proliferation and expression of the prosclerotic cytokine transforming growth factor-β1 (TGF-β1) [22]. Moreover, chronic leptin infusion into normal rats induced proteinuria and focal glomerulosclerosis accompanied by elevated glomerular TGF-β1 and type IV collagen expression [22]. We therefore postulated that the potential paracrine interactions between glomerular endothelial cells and neighboring mesangial cells are responsible for the observed glomerulosclerosis in leptin-infused animals. Given the possible effect of leptin to modulate glucose metabolism in peripheral tissues and the observed effect of leptin to induce TGF-β1 production in glomerular endothelial cells, we investigated in this study the effect of leptin on glucose uptake and extracellular matrix metabolism in cultured mesangial cells derived from the *db*/*db* mouse.

METHODS

Cell culture

Murine mesangial cells were isolated by differential sieving from glomeruli of 12-week-old nondiabetic *db*/*m* and diabetic *db*/*db* mice, as described [23, 24]. The cells stain positive for Thy-1 antigen, desmin, vimentin, and collagen types I and IV [25]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO-BRL, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L glutamine. Cells were passaged every 72 to 96 hours by light trypsinization.

Reverse transcription-polymerase chain reaction

Total RNA (10 μg) isolated from tissues and cultured cells was reverse transcribed using random hexamers (Promega, Madison, WI, USA) in the presence of 500 U reverse transcriptase (Moloney murine leukemia virus; Promega). The resultant cDNA was amplified by polymerase chain reaction (PCR) using the following specific primers for leptin receptors: sense Ob-Ra 5′-AGTGAT CTTTAATTAAAATAGGT-3′, sense Ob-Rb 5′-AGA GAAGTTAGCACTGTT-3′, and a consensus antisense primer for both isoforms of receptors 5′-ATGTCATTG TACCGATAATTA-3′ [26]. A total of 40 amplification cycles was performed, denaturing at 94°C for 30 seconds, annealing at 50°C for 90 seconds, and extending at 72°C for 90 seconds. As positive control using brain tissue, we have confirmed in pilot studies that reverse transcription-PCR (RT-PCR) using the Ob-Rb sense primer and the common antisense primer can specifically detect an Ob-Rb transcript fragment in murine brain cDNA.

2-Deoxy-D-glucose uptake

Measurement of glucose uptake was performed in 12-well plates, using 2-deoxy-D-glucose (2DOG) as substrate by the method of McClain et al [27]. After overnight incubation in serum-free media, cells were exposed to various concentrations of recombinant mouse leptin from 1 to 100 ng/mL (Biomol, Plymouth Meeting, PA, USA) in the presence or absence of 5 to 50 μmol/L LY294002 (Calbiochem, La Jolla, CA, USA), a PI-3K inhibitor. For uptake studies, cells were incubated in Krebs-Ringer phosphate-HEPES buffer (131.2 mmol/L NaCl, 4.71 mmol/L KCl, 2.47 mmol/L $CaC1_2$, 1.24 mmol/L $MgSO_4$, 2.48 mmol/L NaH_2PO_4, 10 mmol/L HEPES, and 0.5% bovine serum albumin (BSA), pH 7.45] with 2-deoxy-[1-^{3}H]-D-glucose (0.4 μCi/well; Amersham Pharmacia, Arlington Heights, IL, USA) and 0.1 mmol/L unlabeled 2DOG for 5 to 15 minutes. Uptake over this time period was nearly linear. Incubation was terminated by rapidly aspirating the buffer and washing twice with phosphate-buffered saline (PBS). Cells were then harvested with 1 mL of 1 N NaOH and were rinsed with 1 mL of 1 N HCl for β-emission counting. Cell protein was determined in duplicate samples from 12-well plates using DC protein assay reagent (Bio-Rad, Hercules, CA, USA).

Northern blot analysis

Mesangial cells were cultured in 75 cm^2 flasks in DMEM containing 10% FBS and treated with various concentrations of leptin. Total RNA was isolated by repeated phenol-chloroform extraction [25, 28], and 25 μg of total RNA were separated on a 1.2% agarose gel containing 0.67 mol/L formaldehyde. After transferring

by capillary blotting to a Gene-Screen nylon membrane (NEN Research Products, Bad Homburg, Germany), the integrity and equal loading of RNA samples were assessed by methylene blue staining [29]. The following cDNA probes were used for hybridization: murine TGF-β1, murine TGF-β type II receptor (TβRII), murine α1(I) collagen, and rat GAPDH [29–31]. All cDNA inserts were separated from their plasmids in low-melt agarose gel and labeled with 50 μCi [^{32}P] deoxycytidine 5′-triphosphate (Amersham Pharmacia) using the Ready-To-Go DNA labeling kit (Amersham Pharmacia). Hybridization and washing were performed as previously reported [30]. The membranes were then autoradiographed with intensifying screens (Kodak, Rochester, NY, USA) at −70°C for one to four days. Exposed films were scanned with a laser-densitometer (Hoefer Scientific Instruments, San Fernando, CA, USA), and all mRNA levels were calculated relative to those of GAPDH. Measurements of ratios in control cells were assigned a relative value of 100%.

Enzyme-linked immunosorbent assay for type I collagen

Mesangial cells were cultured in a 60 mm plastic dish in DMEM with 10% FBS, containing 50 μg/mL L-ascorbic acid and 50 μg/mL β-aminopropionitrile to promote collagen synthesis and prevent cross-linking, respectively. Cells were treated with 1 to 1000 ng/mL leptin in the presence or absence of 5 to 50 μmol/L LY294002 for the indicated time intervals. The conditioned media were collected, centrifuged, and stored at −20°C. Enzyme-linked immunosorbent assay (ELISA) was performed as previously described [32]. In brief, a 96-well plate (Nunc, Naperville, IL, USA) was coated with 150 μL of conditioned media overnight at 4°C. The wells were washed five times with PBS/1% Tween-20 and blocked with PBS/1% BSA for 30 minutes. Rabbit anti-mouse type I collagen antibody (1:200; Chemicon, Temecula, CA, USA) in PBS/1% BSA was used as the primary antibody for two hours at room temperature. After washing, the wells were incubated with 1:4000 peroxidase-conjugated anti-rabbit antibody (Chemicon) in PBS/1% BSA for one hour at room temperature. Final detection was performed by adding peroxidase substrate (Bio-Rad) for 20 minutes, and optical density was assessed at 405 nm. Mouse type I collagen (Chondrex, Redmond, WA, USA) was used as the standard, and linear correlation was found over a dose range from 3 to 200 ng/mL. All results were normalized to the total protein content of the cells.

Statistical analysis

Data are presented as the mean ± SEM, with N as the number of different experiments. Groups were compared by analysis of variance, and individual groups were compared by Student t test. A P value < 0.05 was considered significant.

RESULTS

We focused on the *db*/*db* mesangial cell since this cell type is more likely to encounter increased leptin levels in vivo. In general, qualitatively similar results were obtained in *db*/*m* mesangial cells, but details of these findings are not addressed here.

Expression of leptin receptor isoforms

The differential expression of the leptin receptor isoforms Ob-Ra or Ob-Rb was assessed by RT-PCR that was designed to screen qualitatively for the presence of these isoforms. Figure 1A reveals that Ob-Ra, a short-form of the leptin receptor, was expressed in cultured mesangial cells and in whole kidney of both *db*/*m* and *db*/*db* mice. On the other hand, Ob-Rb, the full-length leptin receptor, was not detectable in mesangial cells or in whole kidney of either *db*/*m* (data not shown) or *db*/*db* mice (Fig. 1B). Thus, in this study, any observed effect of leptin on mesangial cells would most likely be mediated by Ob-Ra rather than Ob-Rb.

Effect of leptin on 2DOG uptake

We measured 2DOG uptake in *db*/*db* mesangial cells after six hours of incubation with leptin (Fig. 2), since our preliminary experiments showed that leptin maximally stimulates glucose uptake at six hours in contrast to the immediate effect of insulin [33]. For the reported studies, the uptake period chosen was 10 minutes of isotope pulse. Leptin doses ranging from 2 to 100 ng/mL significantly increased glucose uptake in the *db*/*db* mesangial cell (Fig. 2), with the maximum increase (83% above baseline) seen at the 10 ng/mL dose. The 20 and 100 ng/mL doses also stimulated 2DOG uptake to a similar degree. In mesangial cells derived from nondiabetic *db*/*m* mice, leptin also increased 2DOG uptake dose dependently (data not shown), with the maximum effect (79% above baseline) seen at the 50 ng/mL dose. We then assessed whether leptin-induced glucose uptake was modified by LY294002, the PI-3K inhibitor. As shown in Figure 3, pretreatment of the *db*/*db* mesangial cells with 5 μmol/L LY294002 totally prevented the stimulation of 2DOG uptake by 100 ng/mL leptin. Importantly, pretreatment with LY294002 did not affect the basal 2DOG uptake in control cells that were not treated with leptin (Fig. 3).

Effect of leptin on TGF-β, TβRII, and α1(I) collagen mRNA expression

Figure 4 reveals that increasing doses of leptin up to 48 hours did not significantly affect the TGF-β1 mRNA level in *db*/*db* mesangial cells. Similar findings were ob-

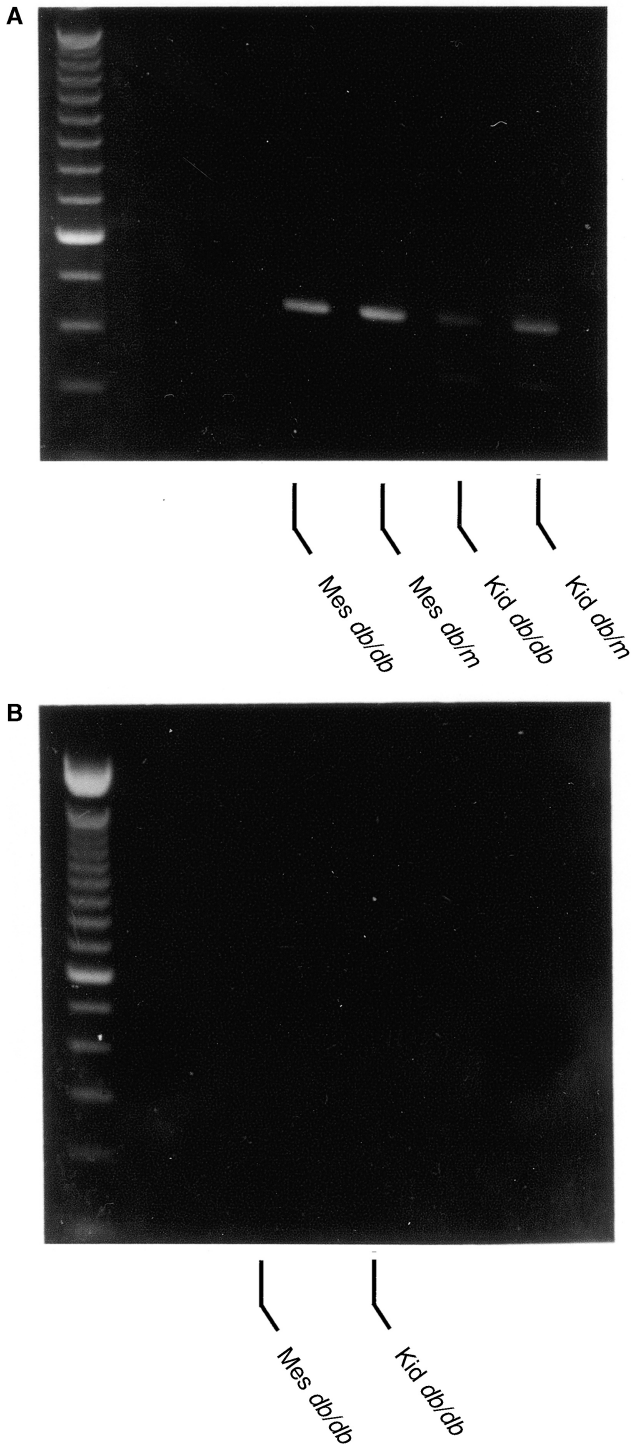

Fig. 1. Leptin receptor isoform expression by RT-PCR in mesangial cells and kidney. (*A*) The short-form leptin receptor (Ob-Ra) is expressed in cultured mesangial cells and whole kidney of both nondiabetic *db*/*m* and diabetic *db*/*db* mice. (*B*) The full-length leptin receptor (Ob-Rb) was not detected in both mesangial cells and whole kidney.

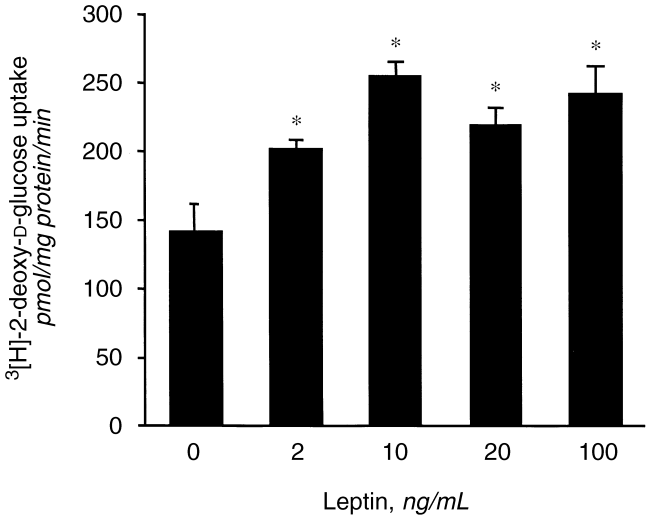

Fig. 2. Dose–response effect of leptin on glucose uptake in *db*/*db* mouse mesangial cells. 2DOG uptake was measured in *db*/*db* mesangial cells treated with various concentrations of leptin for six hours. Data are mean ± SEM, $N = 4$, $^*P < 0.05$ vs. control.

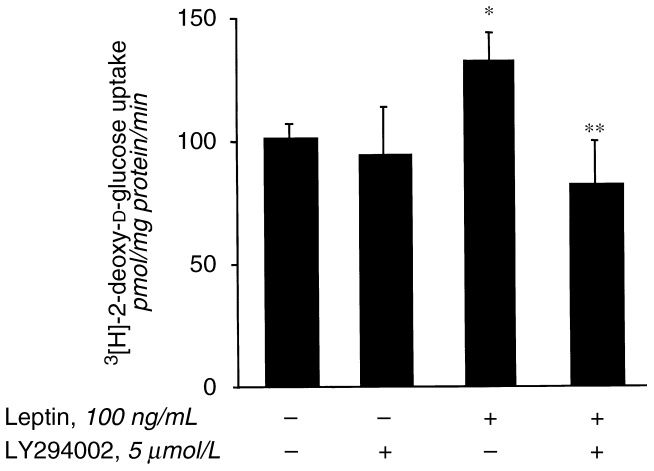

Fig. 3. Effect of phosphatidylinositol-3 kinase (PI-3K) inhibitor (LY294002) on leptin-stimulated glucose uptake in *db*/*db* mouse mesangial cells. Cells were incubated with 100 ng/mL leptin and 5 μmol/L LY294002 for six hours, and 2DOG uptake was measured. Mean ± SEM, $N = 3$, $^*P < 0.05$ vs. control; $^{**}P < 0.05$ vs. leptin treated.

tained with increasing doses of leptin up to 24 hours (data not shown). Furthermore, total TGF-β1 protein production, assessed by ELISA (R&D Systems, Minneapolis, MN, USA), was not increased in the supernatant of cells cultured in 100 ng/mL leptin for 48 hours (2461 pg TGF-β1 per mg protein vs. 2340 pg in control, $N = 4$, P = NS). Whether leptin affects bioactivation of latent TGF-β1 in mesangial cells will require additional studies. In contrast to TGF-β1 mRNA, the TβRII mRNA (encoding the primary ligand-binding signaling receptor for TGF-β) was significantly increased by leptin. This effect was dose dependent (Fig. 5), and was observed as early

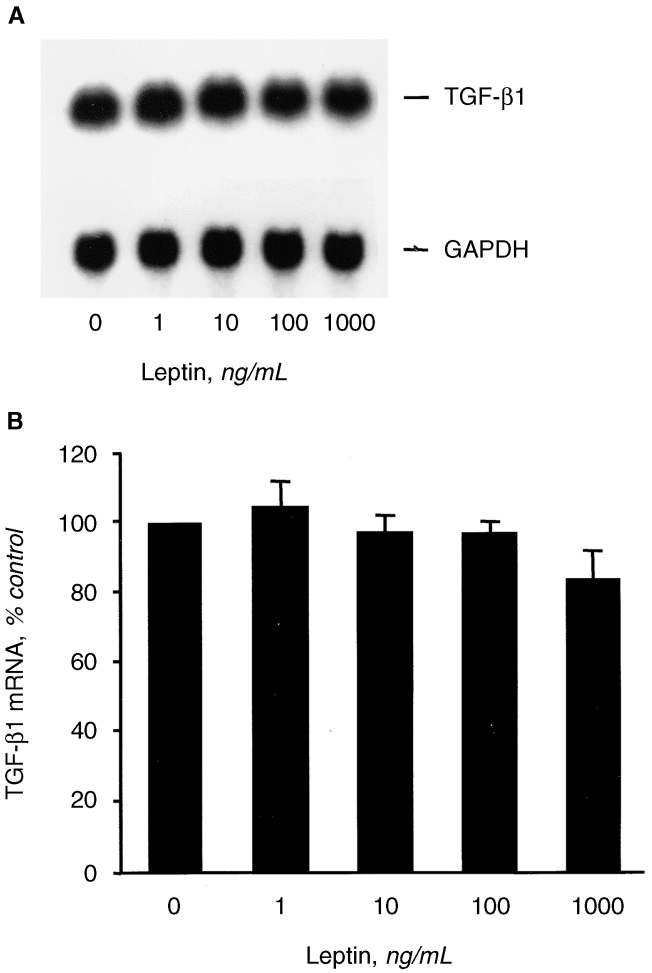

Fig. 4. Effect of leptin on TGF-β1 mRNA expression in *db*/*db* mouse mesangial cells. Cells were treated with various concentrations of leptin (0 to 1000 ng/mL) for 48 hours. Total RNA was analyzed by Northern analysis. (*A*) Representative Northern blot hybridized with TGF-β1 cDNA followed by GAPDH to control for RNA loading and transfer. (*B*) Quantitative results of TGF-β1/GAPDH mRNA ratios. Data are mean ± SEM, $N = 3$, P = NS.

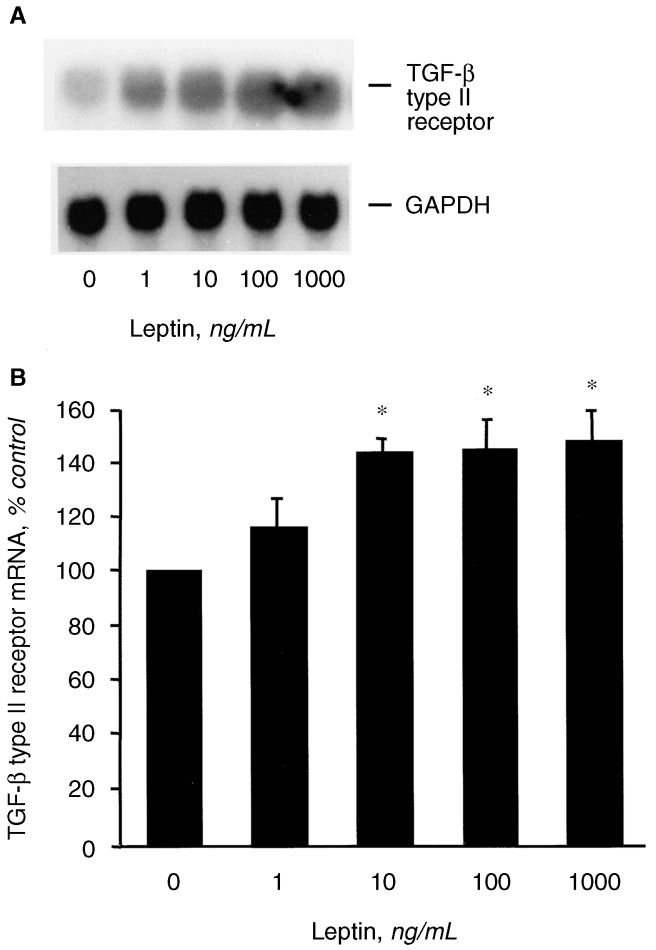

Fig. 5. Effect of leptin on TGF-β type II receptor (TβRII) mRNA expression in *db*/*db* mouse mesangial cells. Cells were treated with various concentrations of leptin (0 to 1000 ng/mL) for 24 hours. Total RNA was analyzed by Northern analysis. (*A*) Representative Northern blot hybridized with TβRII cDNA followed by GAPDH. (*B*) Quantitative results of TβRII/GAPDH mRNA ratios. Data are mean ± SEM, $N = 3$, $^*P < 0.05$ vs. control.

as 12 hours (data not shown) and was persistent at 24 (Fig. 5) and 48 hours (data not shown). Quantitative analysis showed that the maximal stimulation (~50% above control) of TβRII mRNA was achieved at 10 ng/mL leptin. Similarly, leptin treatment of *db*/*db* mesangial cells significantly increased the α1(I) collagen mRNA level, which became maximal at a dose of 10 ng/mL leptin or higher at 48 hours (Fig. 6).

Effect of leptin on type I collagen protein production

To assess type I collagen protein production, ELISA was performed on the supernatants of *db*/*db* mesangial cells grown in the presence of L-ascorbic acid and β-aminopropionitrile. Figure 7 shows that 100 ng/mL leptin significantly stimulated type I collagen protein production compared with control (30% increase at 48 hours, $P < 0.05$, $N = 3$), paralleling the increase in α1(I) collagen mRNA upon leptin treatment. The addition of 50 μmol/L LY294002 diminished the leptin-induced type I collagen protein production (Fig. 7), and this effect of LY294002 was dose dependent (data not shown). At the relatively high dose of 50 μmol/L, LY294002 also inhibited basal collagen production in control cells (Fig. 7).

Effect of leptin and TGF-β1 on type I collagen production

Since leptin stimulates TGF-β1 protein production by glomerular endothelial cells [22], we postulated that the leptin-induced TβRII expression (Fig. 5) in mesangial cells primes them to respond in paracrine fashion to endothelial-derived TGF-β1 by increasing their synthesis of matrix. We therefore assessed the effect of exogenous

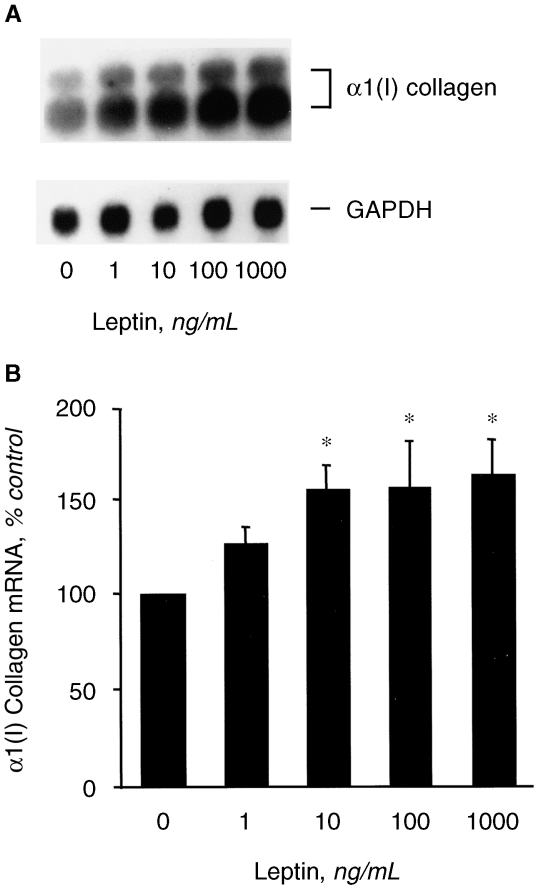

Fig. 6. Effect of leptin on α1(I) collagen mRNA expression in *db*/*db* mouse mesangial cells. Cells were treated with various concentrations of leptin (0 to 1000 ng/mL) for 48 hours. Total RNA was analyzed by Northern analysis. (*A*) Representative Northern blot hybridized with α1(I) collagen cDNA followed by GAPDH. (*B*) Quantitative results of α1(I) collagen/GAPDH mRNA ratios. Data are mean ± SEM, $N = 3$, $^*P < 0.05$ vs. control.

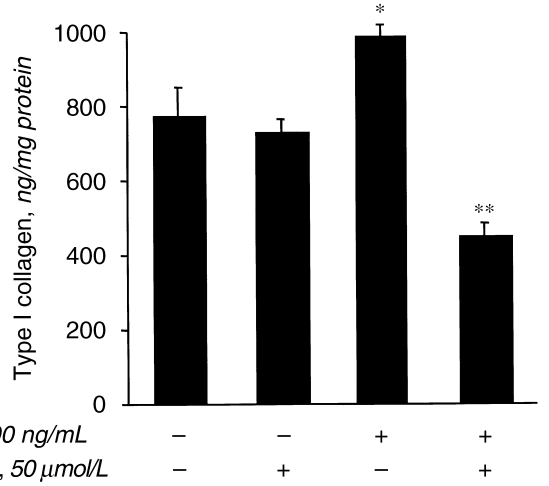

Fig. 7. Effect of leptin on type I collagen protein production. Cells were incubated with or without 100 ng/mL leptin and/or 50 μmol/L LY294002 for 48 hours. Type I collagen protein production was measured by ELISA. Data are mean ± SEM, $N = 3$, $^*P < 0.05$ vs. control; $^{**}P < 0.05$ vs. leptin treated.

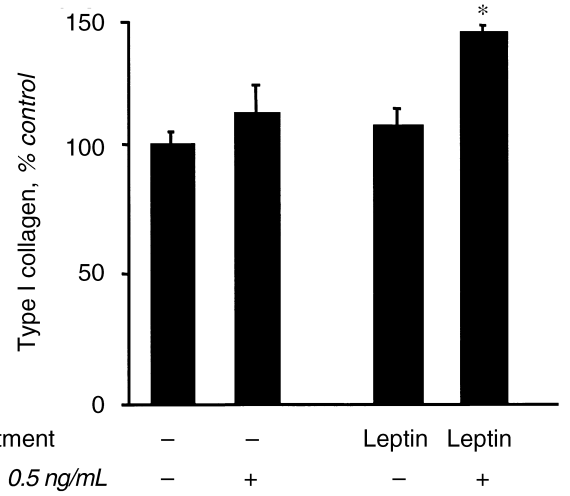

Fig. 8. Response of *db*/*db* mesangial cells pretreated with leptin to exogenous TGF-β1. Cells were pretreated with 100 ng/mL leptin for 24 hours, and media were changed to omit leptin. Cells were then exposed to 0.5 ng/mL TGF-β1 for an additional 24 hours. Protein amount of type I collagen was measured by ELISA. Note that type I collagen was significantly increased by TGF-β1 in leptin-pretreated cells compared with control cells. Data are mean ± SEM, $N = 3$, $^*P < 0.05$ vs. leptin (+), TGF-β1 (−) control.

TGF-β1 on type I collagen production in mesangial cells, with or without leptin pretreatment. After pretreatment for 24 hours with 100 ng/mL leptin (so as to allow for up-regulation of TβRII; Fig. 5), cells were exposed for an additional 24 hours to a submaximal dose of TGF-β1 (0.5 ng/mL) that by itself does not significantly stimulate collagen production. Figure 8 demonstrates a significant increase in type I collagen production (by 45%) in response to TGF-β1 in leptin-pretreated *db*/*db* mesangial cells compared with nonpretreated cells. This result provides indirect evidence that leptin-stimulated TβRII expression in *db*/*db* mesangial cells exaggerates their profibrotic response to exogenous TGF-β1. In fact, Figure 9 shows that leptin and TGF-β1, when added together at their maximal respective doses, exerted additive effects on type I collagen production. Cells exposed to either 100 ng/mL leptin or 2 ng/mL TGF-β1 alone for 48 hours increased type I collagen production by 30 and 36%, respectively (Fig. 9). However, cells exposed to both agents for 48 hours increased type I collagen production by 70% (Fig. 9).

DISCUSSION

The present study demonstrates that a short-form leptin receptor (Ob-Ra), and not the full-length leptin receptor (Ob-Rb), is detectable in murine mesangial cells derived from nondiabetic *db*/*m* and diabetic *db*/*db* mice.

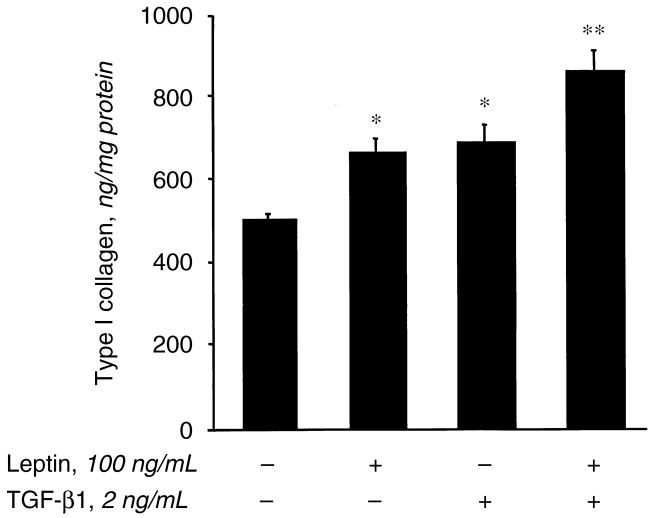

Fig. 9. Additive effects of leptin and exogenous TGF-β1 on type I collagen protein production in *db*/*db* mesangial cells. Cells were incubated with or without 100 ng/mL leptin and/or 2 ng/mL TGF-β1 for 48 hours. Protein amount of type I collagen was measured by ELISA. Data are mean ± SEM, $N = 3$, $^*P < 0.05$ vs. control; $^{**}P < 0.05$ vs. other groups.

Leptin enhances glucose uptake, up-regulates TβRII expression, and stimulates type I collagen mRNA and protein levels in *db*/*db* mesangial cells. The enhanced collagen production may result from the leptin-induced increase in glucose uptake, which is consistent with our finding that leptin-stimulated 2DOG uptake and type I collagen production were inhibited by the PI-3K inhibitor LY294002. Finally, leptin and TGF-β1 have additive effects on type I collagen production in these mesangial cells.

The rationale for conducting this study relates to the results of our previous investigation on the profibrogenic effects of leptin in the kidney [22]. We previously showed that leptin stimulates the glomerular endothelial cell to proliferate, an effect that can be abolished by an antileptin receptor antibody [22]. In addition, incubation of glomerular endothelial cells with leptin stimulates TGF-β1 mRNA and protein levels. Chronic leptin infusions for three weeks into normal rats produced focal glomerulosclerosis and significant proteinuria in association with increased glomerular TGF-β1 expression and type IV collagen accumulation [22]. Thus, our current investigation in mesangial cells and our previous work in glomerular endothelial cells suggest that hyperleptinemia, which occurs in morbid obesity and type 2 diabetes, activates a paracrine TGF-β cross-talk between these neighboring cell types to promote sclerosis in the glomerular compartment.

There are at least five isoforms of the Ob-R receptor system, distinguished by the length of the intracellular domain and the sequence composition that is generated by alternative mRNA splicing [2, 3]. The Ob-R system belongs to a member of the class I cytokine receptor superfamily [34]. The intracellular domain of Ob-Rb contains box-1, -2, and -3 motifs, which can transduce a complete signal via Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathways [35]. On the other hand, Ob-Ra has only a box-1 motif, which was recently found to transmit certain signals [4, 5, 7, 36]. A novel leptin signaling pathway has been delineated involving PI-3K–dependent activation of cyclic nucleotide phosphodiesterase 3B, which inhibits insulin secretion in pancreatic β cells [37]. In C2C12 myotube cells, the leptin-increased glucose transport is mediated by enhanced PI-3K activity [6, 13, 14]. In accord with these studies, we demonstrated that *db*/*db* mesangial cells, in which Ob-Ra but not Ob-Rb is detectable, respond to leptin treatment by significantly increasing glucose uptake and type I collagen production through a PI-3K–dependent pathway.

Recent experimental studies in renal cells have implicated the TGF-β system and the ubiquitous GLUT1 glucose transporter as important pathophysiologic links between hyperglycemia and diabetic nephropathy [38, 39]. TGF-β1 induces glucose uptake by enhancing the expression of GLUT1 in mesangial cells [39]. Overexpression of GLUT-1 in mesangial cells results in increased glucose uptake and extracellular matrix synthesis [40]. Since we demonstrated in the present study that inhibition of PI-3K diminishes leptin-induced glucose uptake as well as type I collagen production, our observations further support a link between glucose uptake and extracellular matrix synthesis in mesangial cells.

The *db*/*db* mouse has a mutation in the full-length receptor isoform Ob-Rb resulting from alternative splicing of the Ob-R mRNA [5]. The high circulating levels of leptin in this mouse (~80 ng/mL) probably result from the loss of a negative feedback loop in the hypothalamus [5]. It is interesting to note that *ob*/*ob* mice that are leptin deficient rarely develop renal disease, but *db*/*db* mice that are hyperleptinemic develop mesangial expansion similar to that of human diabetic nephropathy [21]. Recently, we reported that chronic treatment with the anti–TGF-β antibody prevents the development of the renal lesions in *db*/*db* mice, underscoring the importance of the TGF-β system in mediating diabetic nephropathy in this animal model [41]. In the present study using mesangial cells derived from the *db*/*db* mouse, we show that leptin increases glucose uptake and TβRII expression, as well as augments collagen production especially in response to exogenous TGF-β1. Leptin may thus play a significant role in the overproduction of extracellular matrix through the up-regulation of TβRII, similar to the known effect of high ambient glucose on TβRII in cultured mesangial cells [42]. Previously, we demonstrated that leptin activates TGF-β1 production in glomerular endothelial cells [22]. This leptin-induced TGF-β1 may

stimulate collagen synthesis in neighboring mesangial cells, which are sensitized to TGF-β1 by high leptin levels. These results are consistent with a leptin-mediated paracrine activation of the TGF-β system within the glomerulus that contributes to glomerulosclerosis in the *db*/*db* mouse.

Leptin may also have profibrotic effects on the mesangial cell independent of the TGF-β pathway since inhibition of PI-3K completely abolished the leptin-induced increase in type I collagen and 2-DOG uptake. Furthermore, we found that 100 ng/mL of leptin was equipotent to 2 ng/mL of TGF-β1 in raising type I collagen protein production. Together, exogenous leptin and TGF-β1 at these particular concentrations exerted an additive effect on type I collagen production. These data suggest that leptin and TGF-β1 promote mesangial extracellular matrix expression by different mechanisms. Confirmation of this hypothesis in vivo would require concurrent treatment with both leptin and an effective antagonist of TGF-β such as a neutralizing anti-TGF-β1 antibody. Nevertheless, the two mediators may cooperate and potentiate mesangial matrix production as proposed previously in this article.

A fibrogenic role for leptin in diabetic nephropathy remains to be established, but clinical clues are suggestive. Massively obese patients who have high serum leptin levels [15] tend to develop focal glomerulosclerosis [16]. Type 2 diabetic patients with hyperinsulinemia, who tend to be obese, also exhibit high serum leptin levels [17]. Fruehwald-Schultes et al reported that leptin levels are increased in type 2 diabetic patients with microalbuminuria or macroalbuminuria [19], and Wilson et al reported that the urine leptin concentration in Pima Indians positively correlated with the urinary albumin-to-creatinine ratio and inversely correlated with the glomerular filtration rate [43]. Likewise, with type 1 diabetes, Rudberg and Persson reported that increased serum leptin levels correlated with increased urine albumin excretion in female subjects [18]. Finally, markedly elevated serum leptin levels were found in patients with end-stage renal disease on hemodialysis [44]. Taken together, these observations in conjunction with our results raise the possibility that leptin may cooperate with other mediators to promote increased extracellular matrix production in some forms of renal disease such as diabetic glomerulopathy.

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APPENDIX

Abbreviations used in this article are: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; 2DOG, 2-deoxy-D-glucose; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; JAK, Janus kinase; Ob-R, obese receptor; Ob-Ra, short form leptin obese receptor; Ob-Rb, full-length leptin obese receptor; PI-3K, phosphatidylinositol-3 kinase; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription; TβRII, transforming growth factor-β type II receptor; TGF-β, transforming growth factor-β.

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