Leptin induces TGF-β synthesis through functional leptin receptor expressed by human peritoneal mesothelial cell

JCK Leung[1], LYY Chan[1], SCW Tang[1], KM Chu[2] and KN Lai[1]

[1]Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong; [2]Department of Surgery, Queen Mary Hospital, The University of Hong Kong, Hong Kong

Marked increase in leptin concentration in spent peritoneal dialysate has been reported following continuous ambulatory peritoneal dialysis treatment. The present study was designed to determine whether functional leptin receptor is expressed by human peritoneal mesothelial cells and if so, the possible implication in dialysis. Expression of leptin receptors in cultured mesothelial cells and omental tissue was examined. The effect of leptin on the production of transforming growth factor-β (TGF-β) by mesothelial cells in the presence or absence of high glucose was determined using in vitro culture model of human peritoneal mesothelial cells and adipocytes. The signaling mechanism involved in leptin-induced TGF-β synthesis by mesothelial cells was studied. Both mRNA and protein of the full-length leptin receptor are constitutively expressed in mesothelial cells. The leptin receptor expression in mesothelial cells was upregulated by glucose but not leptin. In adipocytes, glucose increased the mRNA expression and synthesis of leptin. The Janus kinase-signal transducers and activation (JAK-STAT) signal transduction pathway in mesothelial cells was activated by either exogenous or adipocytes-derived leptin. Exogenous leptin induced the release of TGF-β by mesothelial cells. The TGF-β synthesis induced by leptin was amplified by glucose through increased leptin receptor expression. Our novel findings reveal that functional leptin receptor is present on human peritoneal mesothelial cells. The leptin-induced TGF-β synthesis in mesothelial cells is associated with the expression of leptin receptor and the activation of the JAK-STAT signal transduction pathway.

Kidney International (2006) **69**, 2078–2086. doi:10.1038/sj.ki.5000409; published online 26 April 2006

KEYWORDS: leptin; leptin receptor; CAPD; mesothelial cell; TGF-β; JAK-STAT

Correspondence: *KN Lai, Department of Medicine, Room 411, Professorial Block, Queen Mary Hospital, The University of Hong Kong, Hong Kong. E-mail: knlai@hku.hk*

Continuous ambulatory peritoneal dialysis (CAPD) is an important treatment modality of the renal replacement therapy. Unfortunately, the peritoneal membrane frequently exhibits structural and functional changes following long-term dialysis.[1] During CAPD, peritoneal cells are repeatedly exposed to a nonphysiological hypertonic environment that may lead to peritoneal fibrosis and, ultimately, ultrafiltration failure.[2]

Human peritoneal adipocytes (HPAC) are ubiquitously found in peritoneal tissues. There is now compelling evidence suggesting adipocytes can mediate various physiologic processes through secretion of adipokines, including leptin, adiponectin, resistin, tumour necrosis factor-α, interleukin-6, transforming growth factor-β (TGF-β), tissue factors, and other growth factors.[3,4] Adipocytes also express receptors for leptin, insulin growth factor-1, tumour necrosis factor-α, interleukin-6, TGF-β that orchestrate a network of autocrine, paracrine, and endocrine signals.[4] In parietal peritoneum, adipocytes lie deep underneath the mesothelium. During the fluid dwell in CAPD, solutes in peritoneal dialysis fluid (PDF) are transported by passive diffusion through the peritoneal barrier and come into contact with the adipocytes. In the omentum, adipocytes are in close contact with mesothelial cells. Ultrastructural study reveals that a portion of adipocytes are protruded from the mesothelial surface, suggesting that omental adipocytes may be directly exposed to dialysate.[5] In addition, dialysate can also reach the parietal adipose tissue when there is junctional damage or denudation of the mesothelial monolayer. It is therefore logical to postulate that under repeated exposure to PDF and the continuous changes of the physiologic milieu of the peritoneal cavity during CAPD, peritoneal adipocytes will inevitably be 'activated'. Yet, study on any impact of CAPD on peritoneal adipocytes is scarce.

Leptin, an adipocytes-derived 16-kDa hormone, exerts many biological effects through leptin receptor.[4,6] Leptin receptors belong to the class I cytokine family with six spliced isoforms (Ob-Ra to Ob-Rf).[4] Only the full-length isoform, Ob-Rb, contains the intracellular motifs essential for the Janus kinase-signal transducers and activation (JAK-STAT)

signal transduction pathway.[4,7] Accumulating evidence for systemic effects of leptin on specific tissues and metabolic pathways indicates that leptin operates both directly and indirectly to orchestrate complex pathophysiological processes.[6,8] Leptin is cleared principally by the kidney and the serum leptin concentration is increased in patients with chronic renal failure or undergoing dialysis.[9] Marked increase in leptin concentration from serum and spent dialysate has been reported following CAPD treatment.[10] Leptin in peritoneal dialysate is derived not only from plasma but also locally from peritoneal adipose tissue. *In vitro* experiments using adipocyte cell line 3T3-L1 showed glucose-based PDFs induce a higher leptin secretion.[11] In the present study, we determine whether functional leptin receptors are expressed by human peritoneal mesothelial cells (HPMC) and if so, whether these mesothelial leptin receptors could be triggered by leptin to modulate local TGF-β production.

RESULTS

Culture of HPMC and HPAC

Figure 1 shows the cultured cells used in our study. HPMC showed typical cobblestone appearance at confluence (Figure 1a). For *in vitro* experiments, adipocyte differentiation was initiated by culturing preadipocytes (Figure 1b) in adipogenic medium. After 15 days of differentiation, 90% of cells are positively stained with Oil Red O for neutral lipid accumulation (Figure 1C).

Expression of Ob-Rb in omental tissue and cultured HPMC

Expression of the Ob-Rb mRNA (Figure 2a) and protein (Figure 2b) was demonstrated in three different lines of HPMC. Expression of Ob-Rb was confirmed for peripheral blood mononuclear cells and HepG2 cells, which served as positive control for the reverse transcriptase-polymerase chain reaction (RT-PCR).[12,13] Expression of Ob-Rb was detected on cell surface of peritoneal mesothelial cells and adipocytes in omentum (Figure 3). However, HPMC do not produce leptin either constitutively or after incubation with glucose (data not shown).
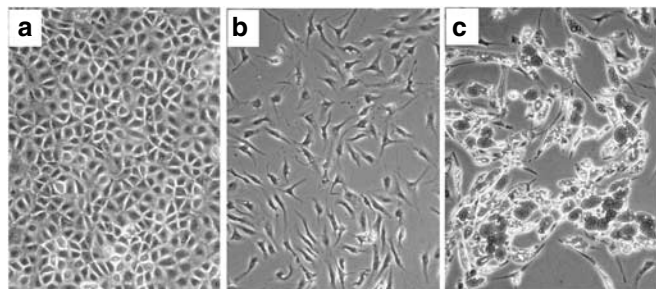

Figure 1 | Culture of peritoneal mesothelial cells and adipocytes. Micrographs showing (**a**) human omental mesothelial cells, (**b**) preadipocytes, and (**c**) differentiated adipocytes in culture. The differentiated adipocytes (HPAC) were positively stained with Oil Red O (original magnification × 100).

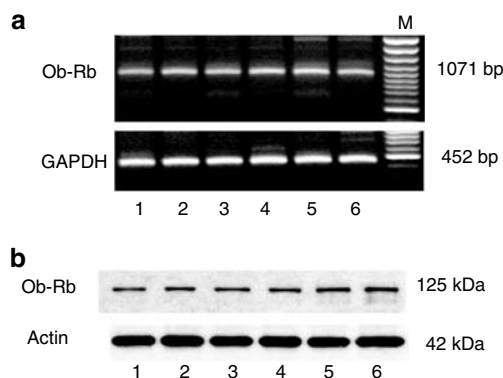**Figure 2 | Expression of Ob-Rb in mesothelial cells.** Expression of (**a**) full-length Ob-Rb mRNA and (**b**) protein in cultured human mesothelial cells. Lanes 1–3 were HPMC cell lines. The liver cell line HepG2 (lane 4), human peripheral mononuclear cells (lane 5), and omental tissue (lane 6) were served as a positive control for Ob-Rb expression.
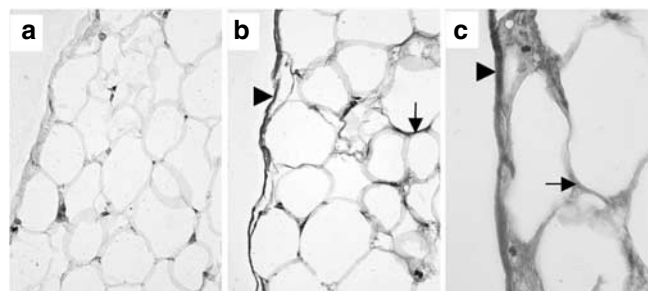

Figure 3 | Immunostaining of Ob-Rb in omental tissue. (**b** and **c**) Representative immunohistochemical staining of Ob-Rb in human omental tissue. Omental mesothelial cells (arrow head) and adipocytes (arrow) showed strong immunoreactivity for Ob-Rb (brown). (**a**) No staining was observed for isotypic control (original magnification (**a** and **b**) × 200, (**c**) × 1000).

Glucose upregulates Ob-Rb expression by HPMC

Figure 4 shows the time course of Ob-Rb expression by HPMC cultured with glucose or mannitol. Expression of Ob-Rb mRNA (Figure 4a) or protein (Figure 4b) was significantly increased in HPMC by incubation with glucose but not with mannitol after 12 h and plateaued at 72 h ($P < 0.005$). Similarly, glucose but not mannitol upregulated Ob-Rb mRNA (Figure 5a) and protein expression (Figure 5b) in HPMC in a dose-dependent manner.

Glucose upregulates leptin mRNA and protein expression by HPAC

Significant increase in mRNA expression of leptin was detected in HPAC after exposed to 40 mM glucose for 12–48 h ($P < 0.005$) (Figure 6a). Then, the leptin expression fell at 72 h and the level remained not different from the baseline value. Glucose significantly increased leptin release from HPAC after exposure for 48 h ($P < 0.005$) (Figure 6b). Glucose at concentration of 20 mM or greater significantly increased the leptin mRNA expression (Figure 7a) and
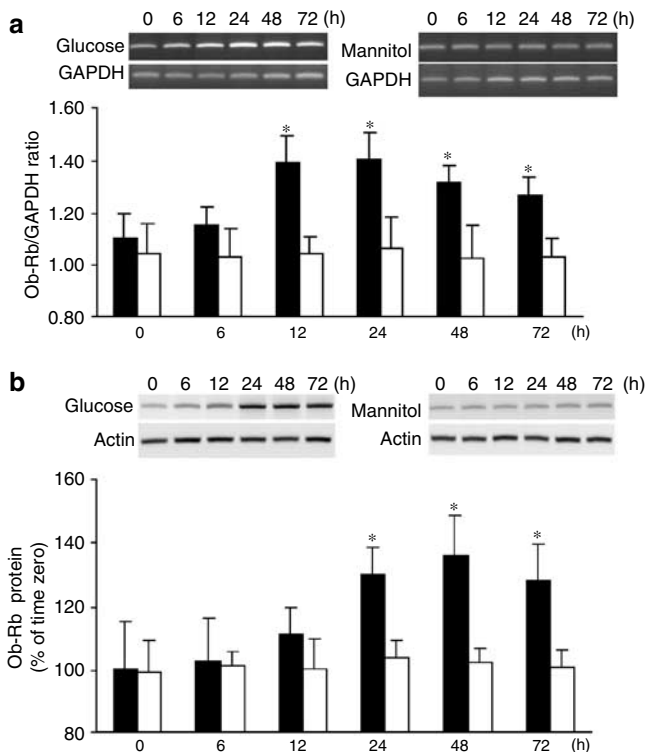
a

b

Figure 4 | Time-dependent upregulation of Ob-Rb expression in HPMC by glucose. Time–response curve for (**a**) Ob-Rb mRNA and (**b**) protein expression in HPMC upon exposure to glucose. Treatment with glucose (40 mM; dark bar) but not mannitol (40 mM; white bar) led to a time-dependent upregulation in Ob-Rb mRNA and protein expression in HPMC. Measurement of Ob-Rb mRNA at time intervals 12, 24, 48, and 72 h; or Ob-Rb protein at time intervals 24, 48, and 72 h differed significantly from values at time zero. Data are mean ± s.d. of four individual experiments. *$P < 0.005$.

a

b

Figure 5 | Dose-dependent upregulation of Ob-Rb expression in HPMC by glucose. Dose–response curve for (**a**) Ob-Rb mRNA and (**b**) protein expression in HPMC upon exposure to glucose for 24 (mRNA) or 48 h (protein). Treatment with increasing concentration of glucose (dark bar) but not mannitol (white bar) led to a dose-dependent upregulation in Ob-Rb mRNA and protein expression in HPMC. Measurement of Ob-Rb mRNA at high concentrations of glucose (20, 40, and 80 mM) differed significantly from the baseline value at 5 mM. Data are mean ± s.d. of four individual experiments. *$P < 0.005$.

protein release (Figure 7b) ($P < 0.005$ for both). Similar upregulation was not observed with mannitol. Expression of Ob-Rb HPAC was not affected by glucose (data not shown).

Leptin did not alter the expression of Ob-Rb but increased the TGF-β release by HPMC

Incubation with increasing dose of leptin (5–40 ng/ml) failed to upregulate the expression of OB-Rb in HPMC (Figure 8a). However, leptin at concentration of 10 ng/ml or greater significantly increased the TGF-β mRNA expression and protein release by HPMC ($P < 0.005$) (Figure 8b).

Activation of JAK-STAT signal transduction pathway in HPMC by leptin

Figure 9 shows the detection of phospho-JAK2 and phospho-STAT3 of the JAK-STAT pathway, phospho-pan-protein kinase (PKC) or *c-fos* in lysate from HPMC following incubation with medium containing glucose (5.6–80 mM), medium containing leptin (0–40 ng/ml), control-HPAC, or glucose-HPAC medium. Increasing concentration of glucose activated the PKC but not the JAK-STAT pathway in a stepwise fashion. In contrast, leptin activated the JAK2, STAT3, and *c-fos* but not PKC in HPMC. Activation of the
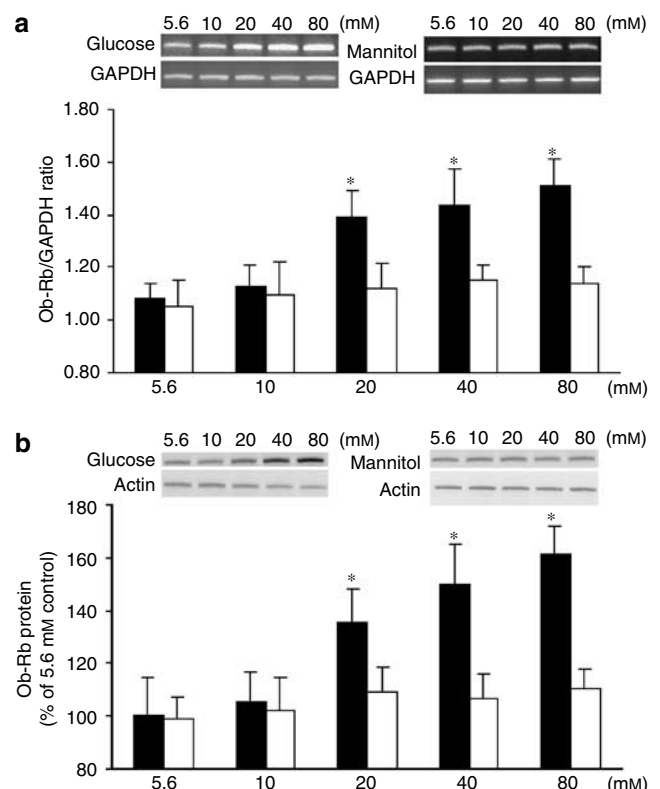
JAK-STAT, *c-fos*, and PKC pathways were detected in HPMC incubated with glucose-HPAC medium but not with control-HPAC medium.

Roles of PKC and JAK-STAT signaling pathways

To study the roles of PKC and JAK-STAT signaling pathways in TGF-β release from HPMC induced by glucose or/and leptin, HPMC was cultured with medium containing 5.6 or 40 mM glucose, 40 mM glucose plus 20 ng/ml leptin, and glucose-HPAC medium in the absence or presence of neutralizing antibody to leptin receptor, inhibitor to JAK2 (AG490; 10 μM), or/and PKC (calphostin; 100 nM). Leptin upregulated TGF-β release by HPMC (Figure 10). Addition of glucose further amplified this TGF-β release. There was synergistic increase (960% of basal value) in TGF-β synthesis by HPMC exposed to 40 mM glucose plus 20 ng/ml leptin. AG490, anti-leptin receptor or calphostin alone only reduced TGF-β release to 575, 637, or 340% of that of the basal level. The TGF-β release in HPMC induced by simultaneous exposure to glucose and leptin was completely abolished only with the presence of both AG490 and calphostin. The
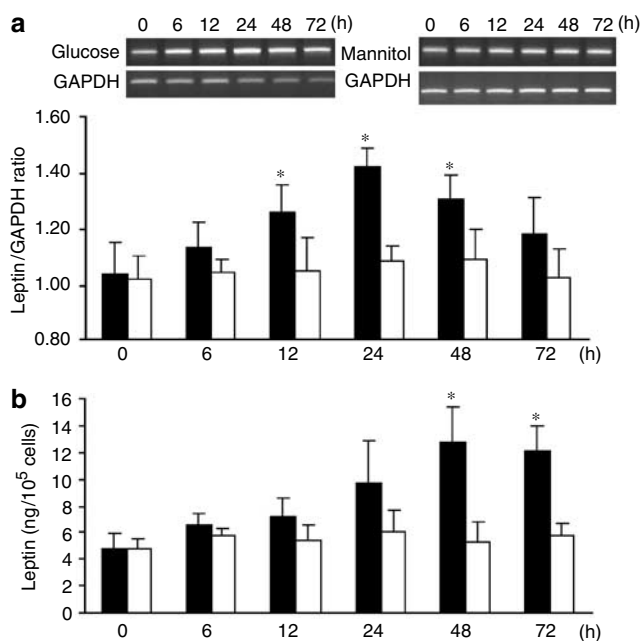
Figure 6 | Time-dependent upregulation of leptin expression in HPAC by glucose. Time–response curve for (**a**) leptin mRNA and (**b**) protein expression in HPAC upon exposure to glucose. Treatment with glucose (40 mM; dark bar) but not mannitol (40 mM; white bar) led to a time-dependent upregulation in leptin gene and protein expression in HPAC. Measurement of leptin mRNA at time intervals 12 h, 24, and 48 h differed significantly from values at time zero. Significance was not observed at 72 h when compared with baseline values. Measurement of leptin protein at 48 and 72 h differed significantly from value at time zero. Data are mean ± s.d. of four individual experiments. *P < 0.005.
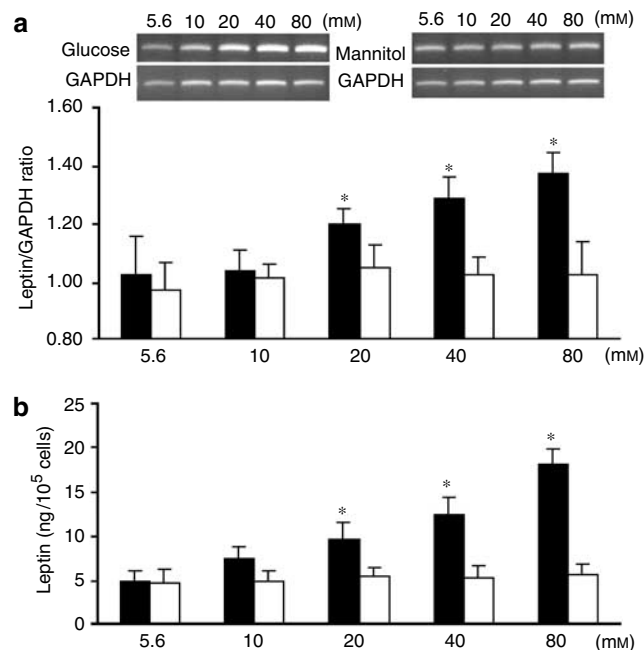
Figure 7 | Dose-dependent upregulation of leptin expression in HPAC by glucose. Dose–response curve for (**a**) leptin mRNA and (**b**) protein expression in HPAC upon exposure to glucose for 24 (mRNA) or 48 h (protein). Treatment with increasing concentration of glucose (dark bar) but not mannitol (white bar) led to a dose-dependent upregulation in leptin gene and leptin release in HPAC. Measurement of leptin mRNA at high-dose concentrations of glucose (20, 40, and 80 mM) differed significantly from baseline value at 5 mM. Data are mean ± s.d. of four individual experiments. *P < 0.005.

concentrations of glucose and leptin in the conditioned medium were 36 ± 2 mM and 9.2 ± 1.4 ng/ml, respectively. Glucose-HPAC medium significantly increased TGF-β synthesis by HPMC (803% of basal level). AG490, calphostin, and antileptin receptor reduced 39.7, 86.96, and 33.1% of the increased TGF-β level, respectively.

DISCUSSION
To the best of our knowledge, this is the first study showing that HPMC express a functional full-length leptin receptor, the Ob-Rb. We have demonstrated that Ob-Rb is constitutively expressed in cultured HPMC and human omental tissue by RT-PCR, immunoblotting, and immunohistochemical staining. The expression of Ob-Rb in HPMC was upregulated following exposure to glucose. The synthesis of leptin by HPAC is upregulated by glucose but not by equimolar mannitol. These data are in keeping with the recent finding that glucose-based PDF induces a higher leptin secretion by murine adipocyte cell line 3T3-L1 compared with dialysate with lower glucose concentration or without glucose.[11]

High glucose content in the dialysate is a major factor causing the structural and functional abnormalities in peritoneal cells during CAPD.[14,15] Decreased expression of intercellular junctional proteins ZO-1 and β-catenin is

observed in HPMC cultured with glucose.[16] Glucose upregulates TGF-β and fibronectin synthesis by HPMC.[17,18] TGF-β is involved in various pathophysiological events in peritoneal biology.[19] Overexpressed TGF-β in murine mesothelial cells results in peritoneal membrane fibrosis and loss of ultrafiltration.[20] In an animal model of peritoneal dialysis, inhibition of TGF-β function and production reduces peritoneal fibrosis.[21] Glucose-induced TGF-β upregulation in kidney mesangial cells and HPMC is mediated by PKC.[18,22] High glucose induces *de novo* synthesis of diacylglycerol and activate PKC in HPMC.[23] Activation of PKC leads to transcription of *c-fos* and *c-jun* early response gene.[24] The *c-fos* and *c-jun* proteins can form dimeric complexes with each other that bind to the activator protein-1 (AP-1) consensus sequence to activate the promoter activity.[25] It has been shown that the AP-1 box B binding sites on the TGF-β promoter mediate the high glucose response in mesangial cells.[26] High glucose increases the TGF-β promoter activity by enhancing the expression of *c-fos*, and hence its binding to AP-1. The involvement of *c-fos* in regulating the TGF-β promoter activity is well demonstrated by the observation that heparin prevents TGF-β activation induced by glucose through reducing the *c-fos* expression, and thereby preventing the formation of activated AP-1 complexes.[27]
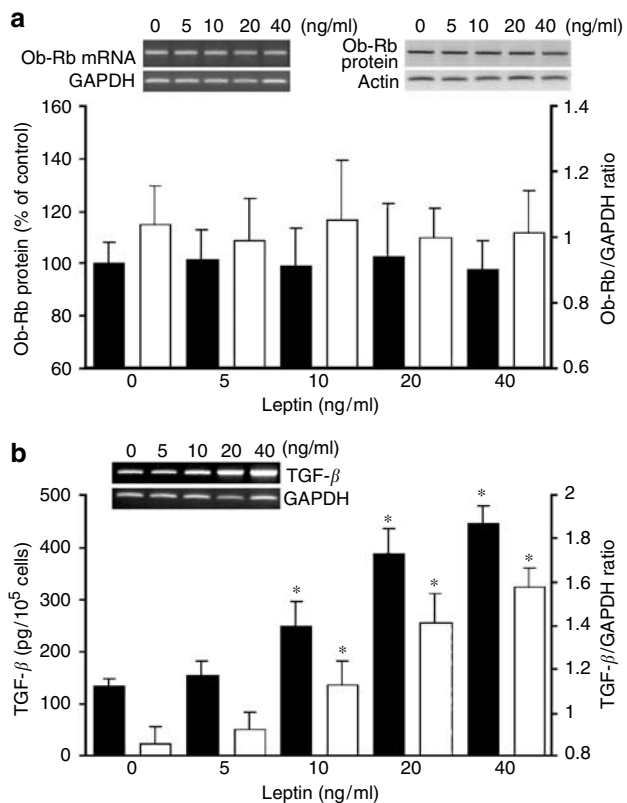
Figure 8 | Effect of exogenous leptin on Ob-Rb expression and TGF-β release by HPMC. (**a**) Expression of Ob-Rb mRNA (white bar) or protein (dark bar) was not altered in HPMC cultured with different concentration of leptin for 24 h (mRNA) or for 48 h (protein). (**b**) Release of TGF-β in HPMC cultured with increasing doses of leptin for 48 h. The expression of TGF-β mRNA (white bar) was significantly increased in HPMC incubated with leptin at 20 and 40 ng/ml. The release of TGF-β (dark bar) was up-regulated in HPMC incubated with leptin at 10, 20, and 40 ng/ml. Data are mean $\pm$ s.d. of four individual experiments. *$P < 0.005$.
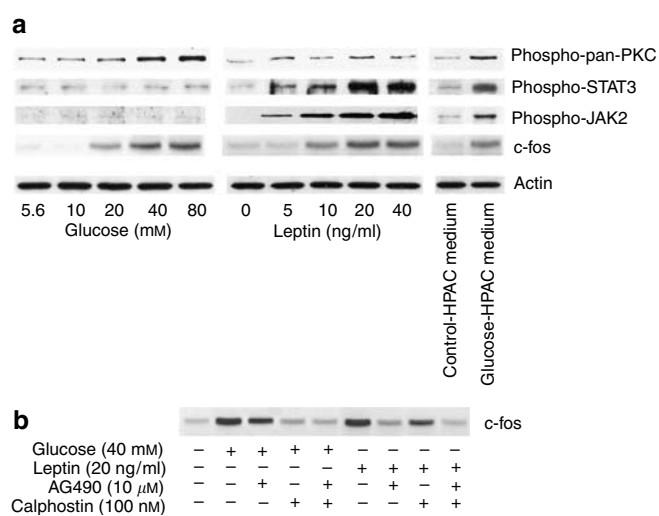

Figure 9 | Detection of different phospho-specific signals in HPMC lysate following incubation with glucose, leptin, glucose-HPAC conditioned medium, or control-HPAC conditioned medium. (**a**) Activation of the PKC signal transduction pathway was detected in HPMC incubated with glucose or glucose-HPAC medium but not with leptin or control-HPAC medium. (**b**) Activation of the JAK2, STAT3, and *c-fos* signal transduction pathways was detected in HPMC incubated with leptin or glucose-HPAC conditioned medium but not with glucose or control-HPAC conditioned medium.

In the present study, we have demonstrated that leptin upregulates the gene expression and protein release of TGF-β in HPMC. HPMC do not produce leptin constitutively or after incubation with high glucose, and there could be a paracrine effect of HPAC-derived leptin on HPMC expression of TGF-β. It had been shown that leptin enhanced aromatase expression in the breast cancer cell line via AP-1.[28] We next examined whether leptin-induced TGF-β synthesis in HPMC is mediated through signal pathways involving PKC and AP-1. Our data showed that PKC is not activated by leptin and neither the leptin-induced TGF-β synthesis is abolished by a PKC inhibitor. Hence, unlike TGF-β synthesis in HPMC induced by high glucose, TGF-β synthesis in HPMC induced by leptin is independent of the PKC pathway. Leptin triggers Ob-Rb signaling through the JAK-STAT signal transduction pathway.[7] In vascular smooth muscle cells, high glucose enhances the angiotensin II-induced cell proliferation through the JAK-STAT pathway.[29] In glomerular mesangial cells, inhibition of the JAK-STAT pathway prevents the glucose-induced TGF-β and fibronectin synthesis.[30] Our immunoblotting data have revealed that leptin, but not glucose, is able to activate JAK2 and STAT3. Furthermore, the TGF-β synthesis in HPMC induced by leptin was abolished by a JAK2 specific inhibitor, AG490. Taken these observations together, TGF-β synthesis in HPMC induced by leptin is triggered by Ob-Rb signaling involving the JAK-STAT pathway. It has been shown that activated STAT3 binds to *c-fos* promoter and upregulates *c-fos* transcription.[31] It is likely that the downstream event in leptin-induced TGF-β synthesis by HPMC is similar to the mechanism of high glucose-mediated TGF-β synthesis, which involves an increased *c-fos* expression and TGF-β promoter activity.

Our data show that in the presence of 40 mM glucose or 20 ng/ml leptin, there was 583 or 349% increase in TGF-β synthesis by HPMC. However, glucose (40 mM) amplified the leptin-induced TGF-β synthesis by HPMC (1113% (glucose + leptin) versus 932% (glucose or leptin $583 + 349 = 932\%$). This amplification of TGF-β synthesis is likely to be due to the increased expression of Ob-Rb in HPMC by high glucose. Indeed, our inhibition studies show that TGF-β synthesis by HPMC in the presence of both glucose and leptin was abolished only by using both PKC and JAK2 inhibitors but not with either one alone. Based on our present data, a hypothetical model of leptin-induced TGF-β synthesis in HPMC is proposed (Figure 11). TGF-β synthesis by HPMC is upregulated by glucose through PKC-dependent mechanisms. HPMC exposed to high glucose will induce PKC activation. PKC will then upregulate the transcription factors *c-fos* and *c-jun*, which form complexes with the AP-1
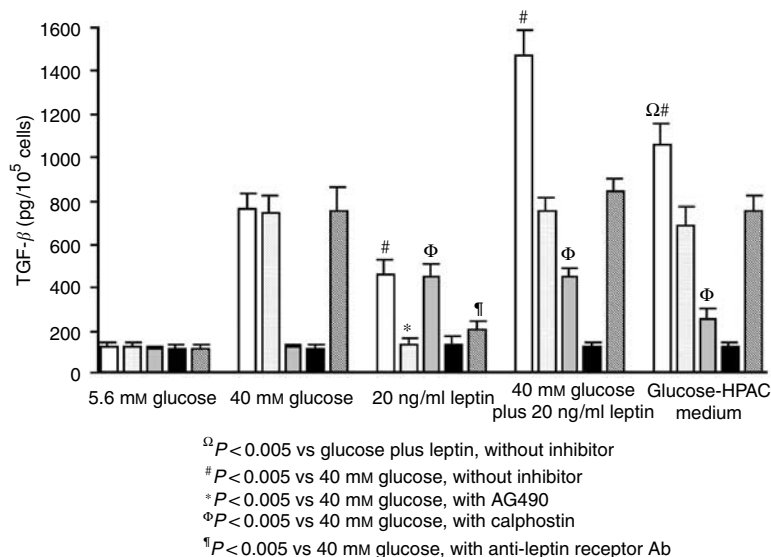
$^{\Omega}P < 0.005$ vs glucose plus leptin, without inhibitor
$^{\#}P < 0.005$ vs 40 mM glucose, without inhibitor
$^{*}P < 0.005$ vs 40 mM glucose, with AG490
$^{\Phi}P < 0.005$ vs 40 mM glucose, with calphostin
$^{\P}P < 0.005$ vs 40 mM glucose, with anti-leptin receptor Ab

Figure 10 | Blocking study of TGF-β release by HPMC. Concentration of TGF-β in culture supernatant from HPMC incubated with medium containing 5.6 mM glucose, 40 mM glucose, 20 ng/ml leptin, 40 mM glucose plus 20 ng/ml leptin, or glucose-HPAC conditioned medium in the absence (white bar) presence of anti-leptin receptor antibody (crossed bar; last bar in each group; 50 μg/ml), inhibitor to JAK2 (AG490; 10 μM; dotted bar; 2nd bar in each group), PKC (calphostin; 100 nM; gray bar) or both (10 μM AG490 plus 100 nM calphostin; dark bar). Data are mean ± s.d. of four individual experiments.
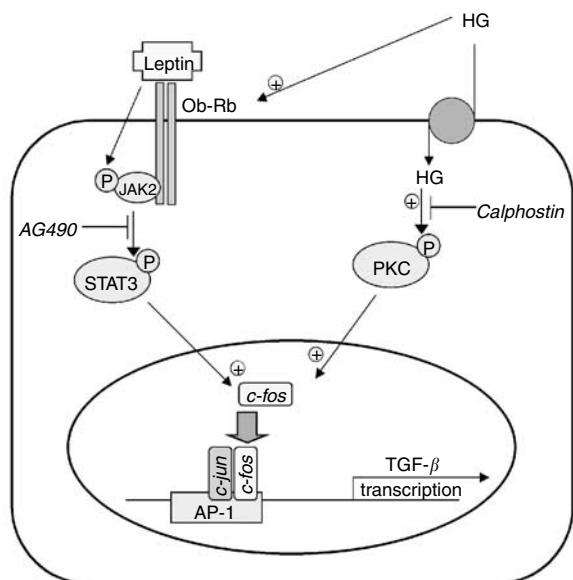

Figure 11 | Hypothetical model of leptin-induced TGF-β synthesis in HPMC. HPMC exposed to high glucose will induce PKC activation. PKC will then upregulate the transcription of early response genes, *c-fos* or/and *c-jun*, which form complexes with the AP-1 binding site and enhance the binding of AP-1 proteins to the AP-1 box B on the TGF-β promoter. TGF-β synthesis is upregualted by high glucose through this PKC-dependent mechanism. Although leptin cannot activate the PKC pathway directly, it does activate the JAK2 and STAT3 through the functional Ob-Rb expressed on HPMC. Activated STAT3 accumulates on the *c-fos* promotor and provides a boost to *c-fos* transcription. High glucose upregulates the expression of Ob-Rb by HPMC as well the PKC-dependent TGF-β synthesis. In the presence of leptin and increased Ob-Rb expression by HPMC stimulated with high glucose, upregulation of TGF-β synthesis will be further amplified through the JAK-STAT pathway.

binding site and enhance the binding of AP-1 proteins to the AP-1 box B on the TGF-β promoter. Although leptin cannot activate the PKC pathway directly, it does activate the JAK2 and STAT3 through the functional Ob-Rb expressed on HPMC. Activated STAT3 accumulates on the *c-fos* promoter and increases *c-fos* transcription. Since high glucose also upregulates the expression of Ob-Rb on HPMC, in the presence of leptin and high glucose, upregulation of TGF-β synthesis by leptin will further be amplified through Ob-Rb and the JAK-STAT3-dependent pathway. Pathways other than JAK-STAT could also be involved in mediating leptin's action. In peripheral blood mononuclear cells, leptin activates the MAPK and phosphatidyl-inositol 3′-kinase pathways, and is implicated in regulating cell growth and glucose metabolism.[32] It has been shown that leptin directly suppressed peroxisome proliferator-activated receptor-γ expression in adipocytes. In kidney mesangial and tubular cells, peroxisome proliferator-activated receptor-γ agonist attenuated high glucose-induced TGF-β release. It is not known whether HPMC expressed peroxisome proliferator-activated receptor-γ and if it is present, the effect of peroxisome proliferator-activated receptor-γ expression on leptin-induced TGF-β release by HPMC. Further examination of these additional pathways in modulating the leptin-induced TGF-β release by HPMC is warranted.

Leptin is elevated during acute infection, in response to proinflammatory cytokines including interleukin-1 and tumour necrosis factor-α.[9] In the kidney, leptin stimulates cell proliferation, collagen IV and TGF-β synthesis in glomerular endothelial cells,[33] and increases the glucose transport, upregulates the expression of TGF-β type II

receptor and collagen I synthesis through phosphatidylino-sitol-3-kinase related pathway in glomerular mesangial cells.[34] These data suggest that leptin triggers a paracrine interaction between glomerular endothelial and mesangial cells through upregulation of TGF-*β* in glomerular endothe-lial cells together with increased TGF-*β* receptor expression in mesangial cells. Whether such paracrine interaction is operating between peritoneal adipocytes and HPMC remains to be explored. Here, we present the first evidence that adipocyte-derived leptin can exert paracrine effect on HPMC through upregulation of Ob-Rb receptor and activation of the JAK-STAT pathway.

It must be emphasized that data obtained from cultured cells model may not exactly reflect *in vivo* situation. During CAPD, mesothelial cells and adipocytes are exposed to un-physiological low pH and high glucose PDF. PDF also contains toxic substances including glucose degradation products. All these factors work together with the cytokines and adipokines networks from peritoneal cells in affecting the TGF-*β* synthesis by mesothelial cells. For the present study, culture model was employed to allow examination of specific variables (high glucose, leptin and Ob-Rb expression), and this will give a preliminary idea of what could be happened *in vivo* during peritoneal dialysis. It is of important to examine the *in vivo* expression of Ob-Rb and related signaling pathway in patients before and under long-term CAPD and future investigation is warranted.

In conclusion, our study provides novel data that leptin can activate the JAK-STAT signal transduction pathway and increase the synthesis of TGF-*β* by HPMC through triggering the functional Ob-Rb receptor. In the presence of high glucose, leptin-induced TGF-*β* synthesis by HPMC is enhanced. Our results implicate that adipocyte-derived leptin may contribute to the peritoneal dysfunction in dialysis through a paracrine effect on mesothelial TGF-*β* synthesis.

MATERIALS AND METHODS
Materials
All reagents for tissue culture were obtained from Invitogen Co (Carlsbad, CA, USA). Monoclonal anti-Ob-Rb, neutralizing anti-leptin receptor antibody and leptin protein were obtained from R&D System (Minneapolis, MN, USA). Monoclonal mouse anti-actin was obtained from Lab Vision (Fremont, CA, USA). Secondary antibodies for immunohistochemical staining and immunoblotting were obtained from Dako (Carpinteria, CA, USA). Antibodies to phospho-JAK2, phospho-STAT3, pan-PKC and *c-fos* were obtained from Cell Signaling Technology (Beverly, MA, USA). All other chemicals were obtained from Sigma (St Louis, MO, USA).

Culture of HPMC
The study was conducted in accordance with the Declaration of Helsinki Principles and was approved by the institutional ethics committee for studies in human. Full informed consent for removing small pieces of omental tissue for experimental studies was obtained from patients. Omentum tissue was obtained from five nonuraemic, nondiabetic patients (male, age 45–55) under elective abdominal surgery for early gastric cancer. The patients have not

been previously exposed to peritoneal dialysis treatment, and were clinically not inflamed. The omental samples collected were further examined histologically to be free of other pathological conditions including inflammation, metastasis, and endometriosis. Mesothelial cells were isolated and characterized using procedures described previously.[35] Once monolayer of HPMC reached confluence (HPMC of second or third passage were used in all experiments), the cells were growth-arrested with serum-free medium for 24 h prior to experiments. Under these conditions, the HPMC remained in a nonproliferative viable condition.

Culture of peritoneal adipocytes
Omental adipocyte precursors from the stromal vascular fraction were isolated by collagenase digestion.[36] For *in vitro* experiments, adipocytes were differentiated by culturing stromal vascular fraction in adipogenic medium (Dulbecco's modified Eagle's medium/Ham's F12 media supplemented with 10 *μ*g/ml transferrin, 100 *μ*M ascorbic acid 2-sodium, 0.85 *μ*M insulin, 20 nM sodium selenite, 0.2 nM triiodothyronine, 1 *μ*M dexamethasone, 100 *μ*M isobutyl-methyl-xanthine, and 1 *μ*M rosiglitazone). The medium was changed 3 days later (dexamethasone and isobutyl-methylxanthine were omitted). After 15 days of differentiation, 90% of cells are positively stained with Oil Red O for neutral lipid accumulation. These differentiated adipocytes were cultured in serum-free M199 medium for 24 h prior to experiment.

Immunohistochemical staining
The expression of Ob-Rb in 4 *μ*m-thicked omental paraffin sections was determined by immunohistochemical staining using mouse anti-Ob-Rb (0.5 *μ*g/ml). The bound antibody was visualised as brown colour using the Dako Envision Plus System (Dako).

Preparation of M199 medium containing mannitol
The basal glucose concentration in M199 medium is 5.6 mM, for all experiment involving the use of mannitol as osmolarity control, the concentration of mannitol added to the M199 medium are 0, 4.4, 14.4, 34.4, and 74.4 mM (plus 5.6 mM glucose in the M199 medium) to generate different sugar concentration (5.6, 10, 20, 40, and 80 mM). For simplicity, these '5.6 mM glucose + mannitol' medium are named as 5.6, 10, 20, 40, or 80 mM mannitol.

Preparation of conditioned medium from HPAC exposed to glucose
HPAC were cultured with M199 medium containing glucose (40 mM) for 48 h. The conditioned medium (glucose-HPAC medium) was stored at −70°C until used. The conditioned media were centrifuged for 10 min at 2000 *g* before experiment. Conditioned medium from HPAC cultured with 5.6 mM basal glucose level (control-HPAC medium) was used as control.

Expression of Ob-Rb by HPMC exposed to glucose
For time–response experiment, HPMC (0.5×10^6 cells) were exposed to 40 mM glucose or mannitol for 0, 6, 12, 24, 48, and 72 h. For dose–response experiment, HPMC were cultured with 5.6, 10, 20, 40, and 80 mM glucose or mannitol for 24 h (mRNA expression) or 48 h (protein release). At any particular time point or at the end of experiment, cells were harvested for total RNA or lysate preparation for determination of expression of Ob-Rb using RT-PCR or immunoblotting.

Expression of leptin mRNA and protein by HPAC exposed to glucose

For time–response experiment, HPAC (1×10^5 cells) were exposed to 40 mM glucose or mannitol for 0, 6, 12, 24, 48, and 72 h. For dose–response experiment, HPAC were cultured with 5.6, 10, 20, 40, and 80 mM glucose or mannitol for 24 h (mRNA expression) or 48 h (protein release). At any particular time point or at the end of experiment, culture supernatants were collected for assay of leptin by ELISA and cells were harvested for total RNA preparation.

Effect of leptin on the expression of Ob-Rb and synthesis of TGF-β by HPMC

HPMC were exposed to 0, 5, 10, 20, and 40 ng/ml leptin for 24 h (mRNA) or 48 h (protein). At the end of experiment, culture supernatants were collected for assay of TGF-β by an ELISA and cells were harvested for total RNA or cell lysate preparations. Ob-Rb or TGF-β mRNA expression was determined by RT-PCR and Ob-Rb protein expression was assayed by immunoblotting.

Activation of JAK-STAT and PKC signal transduction pathways

HPMC/HPAC were exposed to glucose (5.6–80 mm), leptin (0–40 ng/ml), glucose-HPAC medium, or control medium for 30 min. The expression of phospho-JAK2, phospho-STAT3, and phospho-PKC by HPMC was determined using immunoblotting. In another sets of experiment, HPMC were cultured with M199 containing (i) 5.6 mM glucose, (ii) 40 mM glucose, (iii) 40 mM glucose plus 20 ng/ml leptin, or (iv) glucose-HPAC medium in the absence or presence of inhibitor to JAK2 (AG490; 10 μM) and/or PKC (calphostin; 100 nM); or anti-leptin receptor antibody for 48 h (the inhibitors or antibody were added 1 h before experiment started). At the end of experiment, the TGF-β concentration in the culture supernatant was assayed by ELISA.

Gene expression study

Cells were harvested and total cellular RT-PCR was performed as previously described.[37] The hepatocellular line HepG2 was obtained from American Type Culture Collection (ATCC; Rockville, MD, USA) and the human peripheral blood mononuclear cells were isolated from healthy donors by Ficoll gradient separation as previously described.[38] The RNAs obtained from these cells were used as a positive control for Ob-Rb expression. Primers for Ob-Rb, leptin, TGF-β, and glyceraldehyde-3-phosphate dehydrogenase were designed from GeneBank sequences (Ob-Rb U43168; leptin D49487; TGF-β X02812 and glyceraldehyde-3-phosphate dehydrogenase AF261085). The sequences of primers (sense and antisense) are: (i) Ob-Rb, 5′-TCACCCAGTGATTACAAGCT and 5′-CTGGAGAACTCT GATGTCCG; (ii) leptin, 5′-GCTGTGCCCATCCAAAAAGT and 5′-ACTGCCAGTGTCTGGTCCAT; (iii) TGF-β, 5′-GCCCTGGACAC CAACTATTGCT and 5′-AGGCTCCAAATGTAGGGGCAGG; (iv) glyceraldehyde-3-phosphate dehydrogenase, 5′-TGAAGGTCGGAGT CAACGGATTTGGT and 5′-CATGTGGGCCATGAGGTCCACCAC. The PCR products for Ob-Rb, leptin, or TGF-β (1071, 179, or 161 bp) and control (glyuraldehyde-3 phosphate dehydrogenase; 452 bp) were mixed and separated by 1.5% wt/vol agarose gels. The results was analysed using the Gel Doc 1000 Gel Documentation System (Bio-Rad Laboratories Ltd., Hercules, CA, USA).

Immunoblotting

Cells were lysed with buffer containing protease inhibitor cocktails (Sigma). The protein concentrations in cell extracts were measured by a modified Lowry method (DC protein assay kit, BioRad). A total of 10 μg of total protein from the extract was electrophoresed and then transferred onto a polyvinylidine difluoride membrane. The membrane was incubated with mouse anti-Ob-Rb antibody (1:1000), anti-actin antibody (1:1000); rabbit anti-phospho JAK-2 (1:1000), anti-phospho STAT3 (1:1000) or anti-phospho pan-PKC antibody (1:4000). The membrane was incubated with a peroxidase-labeled goat anti-rabbit or anti-mouse immunoglobulin (Dako) before detection with enhanced chemiluminescence (Amersham Pharmacia Biotech, Arlington, IL, USA). The immunoblot images were quantitated using ImageQuant software (Molecular Dynamic, Sunnyvale, CA, USA). Densitometry results were reported as % of control after normalization with the average arbitrary integrated values of the actin signal.

Measurement of TGF-β or leptin in culture supernatant

The concentration of TGF-β or leptin in supernatant was measured by ELISA (R&D System) with a detection limit of 32 or 20 pg/ml and a coefficient of variation of 8.5 or 7.6%, respectively.

Statistical analysis

All data were expressed as means $\pm$ s.d. Intergroup differences between two variables were assessed by the unpaired *t*-test. The data from more than two study groups were analyzed using multivariate analysis of variance followed by Bonferroni correction. All ‘*P*’-values quoted are two-tailed and the significance is defined as $P < 0.005$.

REFERENCES

1. Di Paolo N, Sacchi G, De Mia M *et al.* Morphology of the peritoneal membrane during continuous ambulatory peritoneal dialysis. *Nephron* 1986; **44**: 204–211.
2. Rubin J, Herrera GA, Collins D. An autopsy study of the peritoneal cavity from patients on continuous ambulatory peritoneal dialysis. *Am J Kidney Dis* 1991; **18**: 97–102.
3. Friedman JM. Obesity in the new millennium. *Nature* 2000; **404**: 632–634.
4. Myers Jr MG. Leptin receptor signaling and the regulation of mammalian physiology. *Recent Prog Horm Res* 2004; **59**: 287–304.
5. Di Paolo N, Sacchi G. Atlas of peritoneal histology. *Perit Dial Int* 2000; **20**(Suppl 3): S5–96.
6. Pond CM. Adipose tissue and the immune system. *Prostaglandins Leukot Essent Fatty Acids* 2005; **73**: 17–30.
7. Ahima RS, Osei SY. Leptin signaling. *Physiol Behav* 2004; **81**: 223–241.
8. Fruhbeck G, Gomez-Ambrosi J, Muruzabal FJ, Burrell MA. The adipocyte: a model for integration of endocrine and metabolic signaling in energy metabolism regulation. *Am J Physiol Endocrinol Metab* 2001; **280**: E827–847.
9. Wolf G, Chen S, Han DC, Ziyadeh FN. Leptin and renal disease. *Am J Kidney Dis* 2002; **39**: 1–11.
10. Tsujimoto Y, Shoji T, Tabata T *et al.* Leptin in peritoneal dialysate from continuous ambulatory peritoneal dialysis patients. *Am J Kidney Dis* 1999; **34**: 832–838.
11. Teta D, Tedjani A, Burnier M *et al.* Glucose-containing peritoneal dialysis fluids regulate leptin secretion from 3T3-L1 adipocytes. *Nephrol Dial Transplant* 2005; **20**: 1329–1335.
12. Zarkesh-Esfahani H, Pockley G, Metcalfe RA *et al.* High-dose leptin activates human leukocytes via receptor expression on monocytes. *J Immunol* 2001; **167**: 4593–4599.
13. Liu ZJ, Endoh A, Li R, Ohzeki T. Effects of leptin and dexamethasone on long and short leptin receptor mRNA. *Pediatr Int* 2004; **46**: 561–564.
14. Ha H, Lee HB. Effect of high glucose on peritoneal mesothelial cell biology. *Perit Dial Int* 2000; **20**(Suppl 2): S15–S18.
15. Di Paolo N, Sacchi G. Peritoneal vascular changes in continuous ambulatory peritoneal dialysis (CAPD): an *in vivo* model for the study of diabetic microangiopathy. *Perit Dial Int* 1989; **9**: 41–45.

16. Ito T, Yorioka N, Yamamoto M *et al.* Effect of glucose on intercellular junctions of cultured human peritoneal mesothelial cells. *J Am Soc Nephrol* 2000; **11**: 1969–1979.

17. Noh H, Ha H, Yu MR *et al.* Angiotensin II mediates high glucose-induced TGF-beta1 and fibronectin upregulation in HPMC through reactive oxygen species. *Perit Dial Int* 2005; **25**: 38–47.

18. Ha H, Yu MR, Lee HB. High glucose-induced PKC activation mediates TGF-beta 1 and fibronectin synthesis by peritoneal mesothelial cells. *Kidney Int* 2001; **59**: 463–470.

19. Ihn H. Pathogenesis of fibrosis: role of TGF-beta and CTGF. *Curr Opin Rheumatol* 2002; **14**: 681–685.

20. Margetts PJ, Kolb M, Galt T *et al.* Gene transfer of transforming growth factor-beta1 to the rat peritoneum: effects on membrane function. *J Am Soc Nephrol* 2001; **12**: 2029–2039.

21. Margetts PJ, Gyorffy S, Kolb M *et al.* Antiangiogenic and antifibrotic gene therapy in a chronic infusion model of peritoneal dialysis in rats. *J Am Soc Nephrol* 2002; **13**: 721–728.

22. Haneda M, Koya D, Isono M, Kikkawa R. Overview of glucose signaling in mesangial cells in diabetic nephropathy. *J Am Soc Nephrol* 2003; **14**: 1374–1382.

23. Inoguchi T, Xia P, Kunisaki M *et al.* Insulin's effect on protein kinase C and diacylglycerol induced by diabetes and glucose in vascular tissues. *Am J Physiol* 1994; **267**: E369–E379.

24. Kreisberg JI, Radnik RA, Ayo SH *et al.* High glucose elevates c-fos and c-jun transcripts and proteins in mesangial cell cultures. *Kidney Int* 1994; **46**: 105–112.

25. Halazonetis TD, Georgopoulos K, Greenberg ME, Leder P. c-Jun dimerizes with itself and with c-Fos, forming complexes of different DNA binding affinities. *Cell* 1988; **55**: 917–924.

26. Weigert C, Sauer U, Brodbeck K *et al.* AP-1 proteins mediate hyperglycemia-induced activation of the human TGF-beta1 promoter in mesangial cells. *J Am Soc Nephrol* 2000; **11**: 2007–2016.

27. Weigert C, Brodbeck K, Haring HU *et al.* Low-molecular-weight heparin prevents high glucose- and phorbol ester-induced TGF-beta 1 gene activation. *Kidney Int* 2001; **60**: 935–943.

28. Catalano S, Marsico S, Giordano C *et al.* Leptin enhances, via AP-1, expression of aromatase in the MCF-7 cell line. *J Biol Chem* 2003; **278**: 28668–28676.

29. Morishita R, Gibbons GH, Horiuchi M *et al.* Role of AP-1 complex in angiotensin II-mediated transforming growth factor-beta expression and growth of smooth muscle cells: using decoy approach against AP-1 binding site. *Biochem Biophys Res Commun* 1998; **243**: 361–367.

30. Wang X, Shaw S, Amiri F *et al.* Inhibition of the Jak/STAT signaling pathway prevents the high glucose-induced increase in tgf-beta and fibronectin synthesis in mesangial cells. *Diabetes* 2002; **51**: 3505–3509.

31. Yang E, Lerner L, Besser D, Darnell Jr JE. Independent and cooperative activation of chromosomal c-fos promoter by STAT3. *J Biol Chem* 2003; **278**: 15794–15799.

32. Martin-Romero C, Sanchez-Margalet V. Human leptin activates PI3K and MAPK pathways in human peripheral blood mononuclear cells: possible role of Sam68. *Cell Immunol* 2001; **212**: 83–91.

33. Wolf G, Hamann A, Han DC *et al.* Leptin stimulates proliferation and TGF-beta expression in renal glomerular endothelial cells: potential role in glomerulosclerosis. *Kidney Int* 1999; **56**: 860–872.

34. Han DC, Isono M, Chen S *et al.* Leptin stimulates type I collagen production in db/db mesangial cells: glucose uptake and TGF-beta type II receptor expression. *Kidney Int* 2001; **59**: 1315–1323.

35. Leung JC, Chan LY, Li FF *et al.* Glucose degradation products downregulate ZO-1 expression in human peritoneal mesothelial cells: the role of VEGF. *Nephrol Dial Transplant* 2005; **20**: 1336–1349.

36. Rodriguez AM, Pisani D, Dechesne CA *et al.* Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J Exp Med* 2005; **201**: 1397–1405.

37. Lai KN, Li FK, Lan HY *et al.* Expression of aquaporin-1 in human peritoneal mesothelial cells and its upregulation by glucose *in vitro*. *J Am Soc Nephrol* 2001; **12**: 1036–1045.

38. Chan LY, Leung JC, Tsang AW *et al.* Activation of tubular epithelial cells by mesangial-derived TNF-α: Glomerulotubular communication in IgA nephropathy. *Kidney Int* 2005; **67**: 602–612.