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Original Article

Glucose-containing peritoneal dialysis fluids regulate leptin secretion from 3T3-L1 adipocytes

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Abstract

Background. A marked elevation of serum leptin is observed soon after the start of peritoneal dialysis (PD), suggesting that leptin production may be stimulated by this treatment. Glucose metabolism is the major factor regulating leptin. The current study was designed to test if glucose-based PD fluids might regulate leptin production *in vitro*.

Methods. 3T3-L1 adipocytes were exposed to a 50:50 mixture of dialysis solutions and medium M199 containing 10% serum for $\leq$48 h. Leptin secretion in culture cell supernatants was measured by enzyme-linked immunosorbent assay and leptin mRNA content by northern blot analysis.

Results. The high glucose-based commercial dialysate PD4 produced a higher leptin secretion compared with an identical laboratory-manufactured dialysate (Lab-D), but with a physiological glucose concentration of 5 mM ($P < 0.05$). Raising glucose concentration from 2.75 to 40 mM in Lab-D induced a dose-dependent increase in leptin secretion of $\leq 110 \pm 12\%$ at 48 h ($P < 0.001$) and leptin mRNA ($P < 0.05$; glucose 2.75 *vs* 40 mM). Inhibition of UDP-*N*-acetylglucosamine biosynthesis, with 6-diazo-5-oxo-norleucine added to Lab-D, abolished most of the glucose-stimulated leptin release and downregulated leptin gene expression. Furthermore, glucose-free Lab-D supplemented with 1 mM glucosamine, an intermediate product in UDP-*N*-acetylglucosamine biosynthesis, increased leptin secretion by $28 \pm 11\%$ over control ($P < 0.05$), although without effect on leptin mRNA, after 48 h of culture.

Conclusions. These results suggest that the PD-induced hyperleptinaemia could, in part, be mediated by the effect of glucose-based dialysis fluids on leptin production by adipocytes via activation of the hexosamine biosynthetic pathway.

Keywords: adipocyte cultures; glucose; glucosamine; hexosamine pathway; leptin secretion; peritoneal dialysis fluids

Introduction

The adipocyte hormone leptin signals the body's nutritional status to regulatory centres in the hypothalamus to regulate weight control. Leptin also has distinct cytokine properties, responsible for effects on renal cell growth, haematopoiesis and modulation of the immune system [1], which have stimulated the interest of the nephrologist.

Leptin circulates in proportion to fat mass. However, patients with end-stage renal disease (ESRD) commonly have serum leptin levels several times higher than would be expected for their adipose mass. The major cause of uraemic hyperleptinaemia is reduced renal clearance, although other factors associated with ESRD may contribute to regulate leptin production in this setting. Among patients undergoing renal replacement therapy, those treated by peritoneal dialysis (PD) have extraordinarily raised serum leptin, clearly out of proportion to the fat accumulation observed in these patients [2]. In addition, serum leptin increases by 189% within 1 month after starting PD treatment, in spite of significant leptin removal by the peritoneal route [3]. It is therefore probable that factors other than fat mass stimulate leptin production in PD.

There is abundant evidence that glucose metabolism is the major regulator of leptin production [4]. In PD patients, most dialysis solutions contain high glucose concentrations. It is established that, during the dwell, solutes in PD fluids (especially glucose) are transferred by passive diffusion through the peritoneal

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barrier [5] and, hence, come into contact with omental adipocytes. Leptin is thought to be actively synthesized by these cells, possibly through stimulatory effects by the high glucose environment to which they are exposed [6].

The aim of the present study was, therefore, to investigate the effect of glucose-based PD solutions on leptin secretion and leptin mRNA in cultured 3T3-L1 adipocytes, to determine whether the previously reported strong stimulation of leptin secretion by glucose is still important in the context of dialysis fluids.

Subjects and methods

Dulbecco's modified Eagle's medium (DMEM), M199, fetal bovine serum (FBS) and sodium pyruvate were purchased from Invitrogen (Basle, Switzerland). Penicillin–streptomycin was obtained from Seromed, Switzerland. Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), glucosamine and 6-diazo-5-oxo-L-norleucine (DON) were purchased from Sigma-Aldrich (Basle, Switzerland). Cell culture plastics were supplied by Falcon (Geneva, Switzerland).

Choice of in vitro *model*

In the design of this study, four *in vitro* models of adipose tissue were tested. Human omental adipocytes were isolated by collagenase digestion from omental biopsies from PD patients undergoing catheter insertion. The viable non-adherent adipocyte suspensions obtained were unsuitable, however, as viability declined after only 12 h in suspension. In contrast, pre-adipocytes isolated from the biopsies above were adherent to plastic flasks, proliferated and differentiated in culture. However, the small number of cells available from each tissue donor precluded their use in routine experiments. Omental adipose tissue explants (2–5 mm in diameter) obtained from further donors were also cultured for $\leq$48 h and showed significant, but widely varying, leptin secretion, owing to wide variations in the ratio of adipose to connective tissue in the samples. The present study was, therefore, performed in the 3T3-L1 pre-adipocyte culture model that has been characterized previously for studies of leptin secretion in this laboratory [7,8].

3T3-L1 fibroblasts were grown to confluence and differentiation to adipocytes was stimulated by incubation with IBMX, dexamethasone and insulin, as described previously [7,8]. At this stage, phase-contrast microscopy showed that all the cells exhibited typical adipocyte morphology without any apparent fibroblast contamination. For leptin mRNA studies, cells were treated as reported previously [8].

Leptin protein quantitation

Leptin concentrations in cell culture media were determined using a sandwich enzyme-linked immunosorbent assay for mouse leptin (Quantikine M; R&D Systems, Minneapolis, MN, USA), as described previously [7].

Analysis of leptin mRNA

Total RNA was extracted as described in an earlier study [8]. Leptin mRNA was determined by northern analysis according to standard methods used in this laboratory [9].

Membranes were hybridized with a [^{32}P]dCTP-labelled cDNA probe for mouse leptin, generated as described previously [8]. RNA loading was normalized using a cDNA probe for cyclophilin, which was generously supplied by the Institute of Pharmacology and Toxicology, University of Lausanne, Switzerland.

Experimental design

For leptin secretion studies, 3T3-L1 adipocytes were exposed for $\leq$48 h to a 50:50 mixture of the PD dialysis solution and M199 with 10% FBS containing 500 IU/ml penicillin, 500 µg/ml streptomycin and 0.5 mmol/l sodium pyruvate. The dilution of PD fluid in this way was used to mimic the equilibration of dialysis solutions that occurs early after their infusion into the peritoneal cavity, i.e. a fall in glucose and lactate concentrations and osmolality and an increase in pH [10]. A commercial dialysate with a high glucose concentration (1.36%, PD4; Baxter Healthcare Ltd) or a laboratory-manufactured, filter-sterilized dialysate (Lab-D) of identical electrolyte composition and pH to PD4 were used. The use of the laboratory-made solution enabled the D-glucose concentration of the final test medium to be varied. A 1.36% glucose solution contains 76 mM D-glucose and, hence, after dilution 50:50 with M199 (glucose concentration: 5.5 mM), the test medium has a final glucose concentration of 40.75 mM. Glucose concentrations in Lab-D were varied between 2.5 and 40 mM, allowing assessment of the effect of glucose over a pathophysiological concentration range. Mannitol was used in place of glucose in Lab-D as an osmotic control (Mann) to match PD4's osmolality.

Aliquots of cell supernatants were collected at 24 and 48 h. The samples were frozen at -20°C for subsequent leptin assays. Cell monolayers were lysed by scraping the cells from the culture plate into 0.5 M sodium hydroxide. Total protein was measured using a modified Lowry technique (BioRad DC protein assay; BioRad).

For leptin mRNA experiments, the cells were treated as above for 12, 24 and 48 h. After each of these time points, 1.5 ml phenol guanidine isothiocyanate reagent (Trizol®; Invitrogen) was added. The flasks were subsequently stored at -20°C prior to RNA extraction.

Investigation of the hexosamine pathway

The hexosamine biosynthetic pathway, which is known to use the inwards intracellular glucose flux to regulate leptin production [11], was investigated in two ways: (a) the addition of DON, an inhibitor of the regulatory enzyme glutamine:-fructose-6-phosphate amidotransferase (GFAT), to glucose-based Lab-D in order to reduce the flux through this pathway; and (b) the use of D-glucosamine (2 amino-2-deoxy-D-glucose) that, after transport into the cell and phosphorylation, acts downstream of GFAT and, hence, increases the flux through this pathway. Glucosamine is supplied as hydrochloride salt, which caused a dose-dependent fall in pH in preliminary experiments. As acidosis *per se* decreases leptin production in this cell line [7,8], the pH of the glucosamine-supplemented media was adjusted by the addition of $NaHCO_3$. A further problem is that glucose and glucosamine may compete for the same plasma membrane transporter (GLUT-4), potentially

blunting effects of glucosamine. Experiments were, therefore, conducted in glucose-free dialysis solutions with 1 mM pyruvate as an alternative energy source. Under these conditions, the percentage of lactate dehydrogenase (LDH) released was unaffected (see below) and leptin concentration in the medium was greater at 48 h than at 24 h, demonstrating the continued viability of the cells.

Assessment of cell viability

Cell viability was assessed by measurement of LDH activity released into the cell culture supernatant using a commercial spectrophotometric assay (DG1340-K; Sigma). Cytotoxicity was expressed as the LDH activity in culture supernatant as a percentage of total LDH activity in the cells.

Statistical analyses

Data were expressed as means ± SEM. For comparison of means between two groups, an unpaired *t*-test was used. Comparison of means between multiple groups was by analysis of variance with Duncan's multiple range test. Statistical significance was defined as $P < 0.05$.

Results

Effect of PD4 on leptin secretion

The high glucose-containing dialysate PD4 caused significantly higher leptin release at 48 h, compared with Lab-D containing a physiological glucose concentration of 5 mM. In contrast, the osmotic control Mann did not produce the same effect (Figure 1).

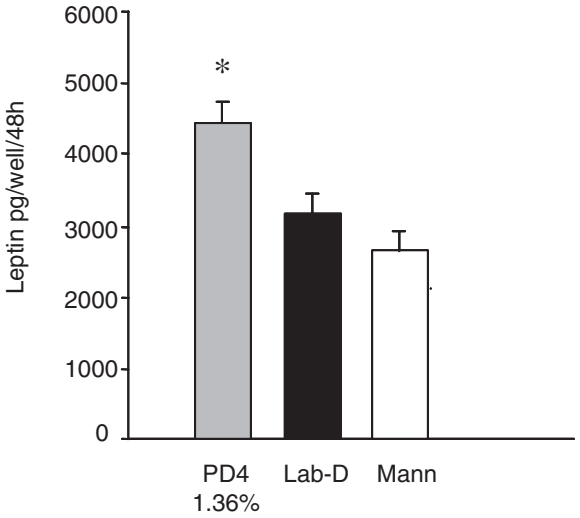

Fig. 1. Effect of different PD fluids on leptin secretion. 3T3-L1 adipocytes in six-well plates were incubated for 48 h in PD4 (glucose 1.36%) or laboratory-manufactured dialysates (Lab-D) identical to PD4 but with a glucose concentration of 5 mM. Mannitol (Mann) was used in laboratory-manufactured dialysate as an osmotic control. Solutions were diluted 50:50 with M199. Results shown are means ± SEM of four independent experiments (pool of 18–23 culture wells for each set of conditions). $*P < 0.05$ *vs* Lab-D.

Effects of glucose concentration on leptin in laboratory-manufactured dialysates

Increasing the glucose concentration from 2.75 to 40 mM in Lab-D increased leptin concentration in the cell supernatants by ≤110 ± 12% in a dose-dependent fashion at 48 h ($P < 0.001$) (Figure 2). Leptin mRNA content, as assessed by northern blot analysis, was also upregulated ($P < 0.05$; glucose 20 and 40 *vs* 2.75 mM). Although there was a trend towards an increase of leptin mRNA at 12 h, the effect became significant at 24 h (Figure 3), preceding the effect on secretion, which in turn reached statistical significance at 48 h. This time sequence strongly suggests that the leptin-stimulating effect by glucose-based PD fluids may be mediated through a transcriptional effect. To test for a possible effect of sterilization technique on PD fluid-induced leptin secretion, the response of cells exposed to commercially produced PD4 (heat sterilized) was directly compared to that with Lab-D (filter sterilized) containing a similar glucose concentration (40 mM). No differences were found ($n = 3$, pool of 14–16 wells for each condition; data not shown).

Finally, the intracellular protein content of the cell lysates was not significantly affected by any of the dialysates tested. Therefore, the observed changes in leptin protein secretion and gene expression could not be accounted for by non-specific effects on cell growth. Moreover, neither PD4 nor any laboratory-manufactured (glucose- or mannitol-based) solutions were associated with significant cell toxicity, as assessed by the percentage of LDH release (data not shown).

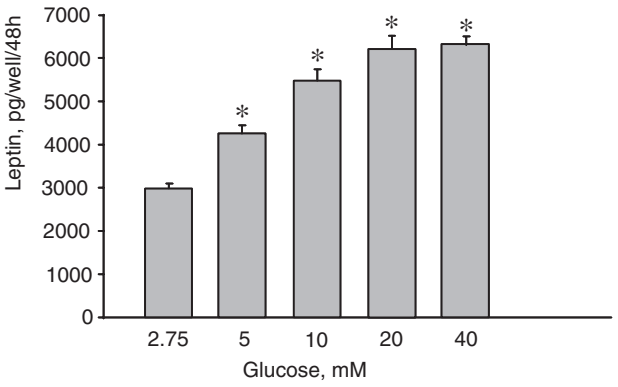

Fig. 2. Effect of glucose concentration in dialysates on leptin secretion. 3T3-L1 adipocytes in six-well plates were incubated in Lab-D at various glucose concentrations. Solutions were diluted 50:50 with M199. The final glucose concentration in the test media was as shown. Results shown are those obtained at 48 h of incubation (means ± SEM of three independent experiments; pool of 22–26 culture wells for each set of conditions). $*P < 0.001$ *vs* 2.75 mM glucose.

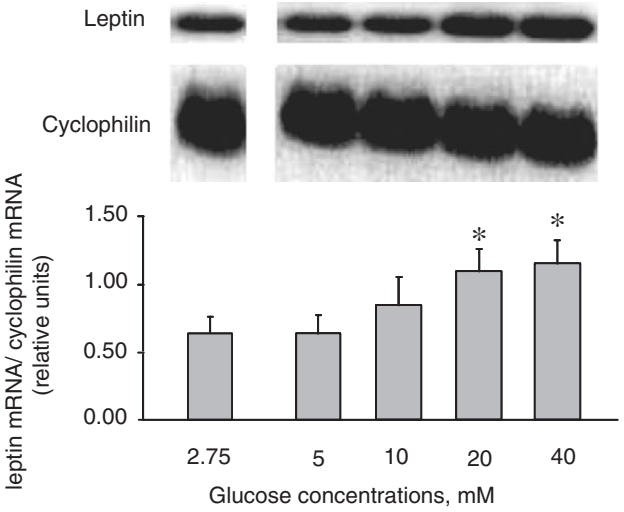

Fig. 3. Effect of increasing glucose concentration in dialysates on leptin mRNA. 3T3-L1 adipocytes in 25-cm^2 flasks were incubated in Lab-D at various glucose concentrations. Solutions were diluted 50:50 with M199. The final glucose concentration in the test media was as shown. Leptin mRNA was assessed by northern blot. Results show a representative blot from three separate experiments at 24 h of incubation. *$P < 0.05$ *vs* 2.75 mM glucose.

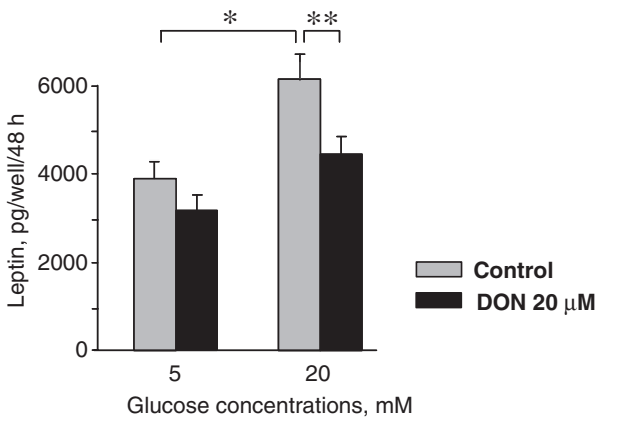

Fig. 4. Effect of the addition of DON in laboratory-manufactured dialysates on leptin secretion. Cells were incubated for 48 h in Lab-D diluted 50:50 with M199. The final glucose concentration in the test media was as shown. Results shown are those obtained at 48 h of incubation (means ± SEM of three independent experiments; pool of 18–20 culture wells for each set of conditions). *$P < 0.05$, 5 *vs* 20 mM glucose. **$P < 0.05$, DON *vs* control (20 mM glucose).

Modulation of the hexosamine biosynthetic pathway

The hexosamine biosynthetic pathway has been proposed as a nutrient sensor linking the incoming glucose flux with leptin gene expression and secretion [11]. Whether this pathway is still involved in the presence of unphysiological PD fluids was therefore determined.

DON (20 μmol/l), added to Lab-D with a high glucose concentration of 20 mM, significantly reduced leptin secretion at 48 h, abolishing most of the leptin-stimulating effect of glucose (Figure 4). Under identical conditions, the addition of DON to Lab-D also decreased leptin mRNA over a 48 h time course (Figure 5). This effect could not be accounted for by cell toxicity, since LDH release in the cell supernatants was insignificant.

High glucosamine concentrations of 10 and 20 mM added to Lab-D suppressed leptin secretion as shown previously [12]. In contrast, D-glucosamine at low doses of 0.1 and 1 mM significantly increased leptin secretion over control by 24 ± 8% and 28 ± 11%, respectively, at 48 h (Figure 6) in Lab-D with no extracellular glucose. However, the addition of D-glucosamine did not affect leptin mRNA, suggesting that, under these conditions, the glucosamine-induced effect on leptin occurs at a post-transcriptional level (Figure 7).

Discussion

Effects of glucose dialysate

It is well established that, in patients treated by PD, the solutes in PD fluids (including glucose) are transferred by passive diffusion through the peritoneal barrier [5]. As a result, cells located in the submesothelial space, e.g. resident fibroblasts and macrophages, have been shown to be activated by solutes from PD fluids [13]. We therefore hypothesized that adipocytes from the omentum, which are in intimate contact with the mesothelial cell layer, also interact with chemical components from PD fluids, especially glucose. The precise glucose concentration experienced by omental adipocytes *in vivo* in patients treated with high glucose PD fluids is unknown, but it is certainly high. The evidence for this is that the extracellular glucose concentration is significantly elevated even at sites well beyond the submesothelial space and, consequently, systemic hyperglycaemia is readily detectable in patients using glucose-based PD fluids [14]. The resulting high glucose concentration in the vicinity of omental adipocytes is regarded as sufficient to stimulate leptin secretion in such patients [6].

This study provides the first evidence that glucose-based dialysis solutions do, indeed, stimulate leptin production from cultured adipocytes (Figure 1), an effect that is attributable to glucose (Figure 2) but not to hyperosmolarity (Figure 1) or to agents generated by heat sterilization of the dialysate. This marked effect of glucose on leptin secretion was obtained in spite of the known inhibitory effect of the low pH [7,8] that occurs in PD solutions.

The hexosamine pathway and glucose dialysate

As the flux through the hexosamine pathway of glucose metabolism has been suggested previously as a mediator of the effect of glucose on leptin synthesis and secretion [11], we tested whether modulation of this pathway could still affect leptin protein release and leptin mRNA in the context of dialysis fluid.

DON, which reduces hexosamine pathway flux by inhibition of GFAT, was shown nearly to abolish the leptin-stimulating effect induced by high

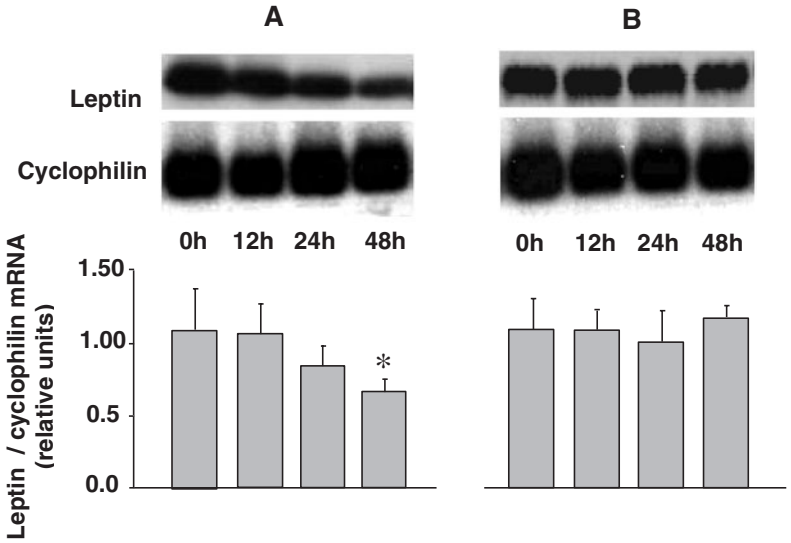

Fig. 5. Effect of the addition of DON in laboratory-manufactured dialysates on leptin mRNA. Cells were incubated for ≤48 h in Lab-D diluted 50:50 with M199 with a final glucose concentration of 20 mM. In Panel A, DON at 20 μM was added to the test medium, whereas in panel B, experiments were conducted without DON (control). Leptin mRNA was assessed by northern blot. Representative blots are shown. Numerical results are means of six culture flasks pooled from two independent experiments. $*P < 0.05$ *vs* 0 h.

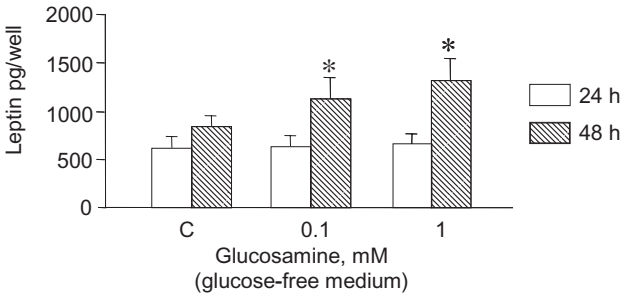

Fig. 6. Effect of glucosamine in laboratory-manufactured dialysates on leptin secretion. Cells were incubated for 48 h in glucose-free Lab-D diluted 50:50 with glucose-free DMEM. Glucosamine was added to test media (concentrations as shown) and leptin release was measured over control (C). Results shown are means ± SEM of four separate experiments (pool of 8–22 culture wells for each set of conditions). $*P < 0.05$ *vs* control.

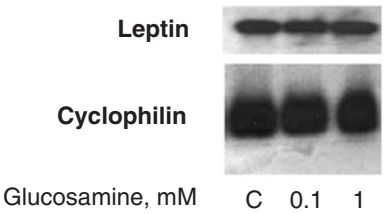

Fig. 7. Effect of glucosamine in laboratory-manufactured dialysates on leptin mRNA. Cells were incubated for 48 h in glucose-free Lab-D diluted 50:50 with glucose-free DMEM, with glucosamine (concentrations as shown) or without (C). Results show a representative blot from four separate experiments.

glucose-based dialysates. This was associated with a decrease in leptin mRNA, suggesting that GFAT modulates leptin production through effects on transcription or stability of leptin mRNA. To test the hypothesis that increasing the metabolic flux into the hexosamine pathway would result in increased leptin production, we incubated cultured adipocytes in Lab-D supplemented with glucosamine. Under conditions designed to avoid potential interference through artefactual glucosamine actions (fall of pH and ATP depletion), glucosamine significantly increased leptin secretion over the control value at 48 h.

Our data in part confirm previous studies that investigated this issue [15,16]. However, the current study has examined these effects specifically in the context of PD fluids. Low pH, high lactate and high osmolarity in dialysates inhibit cell function in many cell types [10]. Therefore, the maintenance of leptin responses to subtle modulations of the hexosamine pathway (Figures 4–7) in the presence of dialysate is noteworthy. The only discrepancy was that glucosamine had no effect on leptin mRNA, which is not in agreement with Zhang *et al.* [16], who demonstrated that glucosamine increased leptin gene promoter activity in 3T3-L1 adipocytes. It should be emphasized that, to avoid unwanted side effects, glucosamine was used in the present study only at a fairly low dose yielding a stimulation of leptin secretion considerably smaller than that observed with high concentrations of glucose. We have shown previously in studies with glucose transport inhibitors in these cells [8] that large changes in glucose influx lead to decreases in both leptin secretion and leptin mRNA, whereas more moderate inhibition of glucose influx decreases leptin secretion without detectable effect on mRNA levels. In response to moderate changes in glucose or glucosamine flux, a non-transcriptional mechanism may be responsible for changes in leptin secretion, possibly through effects at the level of translation. Reports that

the hexosamine pathway and its resulting *O*-Glc-NAc glycosylation of the eukaryotic initiation factor-2 binding protein p^{67} can initiate translation [17] support this view.

Limitations of the present culture model

Incubation conditions. In clinical practice, PD fluids have unphysiologically high glucose concentrations; however, these concentrations are not sustained in the peritoneal cavity since they rapidly fall to 38% of initial values at 4 h [10]. For these reasons, our experiments were restricted to the pathophysiological range of 2.5–40 mM final glucose concentration. The stimulating effects of glucose-based dialysates were seen at 24 h (leptin mRNA) and 48 h (leptin protein release) and these incubation times are longer than the dwell times in PD. It must be emphasized that even the best study design employing cultured cells *in vitro* may not exactly mimic clinical practice. However, potential effects seen *in vitro* at 24 and 48 h may be relevant, since patients on PD repeatedly replace the dialysate with a fresh solution.

Choice of cell line. As a clonal cell line, 3T3-L1 cells have the advantage of a homogeneous cell population allowing precise comparisons of defined treatments, without the confounding factor of variation between different donors. Nevertheless, the 3T3-L1 preadipocyte model (like all *in vitro* models) has limitations in studying the response of omental adipocytes to PD fluids. This is a mouse rather than human cell line and has been used previously as a model of subcutaneous rather than visceral (omental) adipocytes [18]. However, the endocrine effects of cell lines (including 3T3-L1) *in vivo*, when implanted in various fat depots, vary considerably depending on the location of implantation [19]. This means that the *in vivo* environment rather than the nature of the adipocyte cell line *per se* is the crucial factor. It is not certain, therefore, that adipocytes from other sources (e.g. visceral adipocytes from PD patients), if grown *in vitro* in isolation, would have provided a more accurate model than 3T3-L1 adipocytes. Furthermore, omental adipocytes are more leptin-responsive to nutrient stimuli *in vivo* than are subcutaneous adipocytes [20]. Therefore, even if 3T3-L1 do behave more like subcutaneous adipocytes than visceral adipocytes [18], this may mean that the glucose effect observed simply underestimates the effect that would be seen in visceral adipocytes. It has also been shown that leptin responses to changes in glucose availability are qualitatively similar in rat [4], human subcutaneous [15] and 3T3-L1 adipocytes [16], suggesting that marked qualitative differences between cell lines in the glucose response of leptin are unlikely.

It should be emphasized, however, that results obtained with any single cell line must be interpreted with caution and it will, therefore, be interesting to see whether results like those in the present study are also observed in future studies with human adipocyte lines.

In summary, this study provides evidence that glucose contained in dialysis fluids stimulates leptin production from cultured adipocytes and that this effect is at least partly mediated through the hexosamine biosynthetic pathway. The data support the notion that leptin is actively synthesized by intra-abdominal adipose tissue of patients undergoing PD, thus, contributing to sustained hyperleptinaemia associated with this treatment.

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Conflict of interest statement. None declared.

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