

Acidosis Downregulates Leptin Production from Cultured Adipocytes through a Glucose Transport–Dependent Post-transcriptional Mechanism

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Abstract. Metabolic acidosis, a common feature of uremia, has a well documented wasting effect on skeletal muscle. In contrast, the effect of metabolic acidosis on adipose tissue is unknown. Serum levels of the adipocyte hormone leptin have been shown to be lower in acidotic uremic rats when compared with uremic controls. This study investigated the effect of acidosis on leptin protein secretion and leptin gene expression. This was studied *in vitro* by means of 3T3-L1 cultured adipocytes. Leptin secretion was decreased at an acid pH of 7.1 compared with a control pH of 7.5 (1277 versus 1950 pg/well/48 h, $P < 0.05$). In contrast, acidosis did not affect leptin mRNA content. Glucose transport was reduced by 39% at pH 7.1 at 24 h, which was comparable in magnitude with the

inhibition of leptin secretion at the same pH. The glucose transport inhibitors cytochalasin B (0.5 to 50 μM) and phloretin (0.05 to 0.25 mM) mimicked the effect of acidosis and reduced leptin secretion in a dose-dependent fashion ($P < 0.02$). Dose-response curves for the inhibition of glucose uptake showed that decreasing glucose transport to the same extent as with acid was sufficient to drive down leptin secretion, independently of changes of leptin mRNA. Acid decreases leptin secretion from 3T3-L1 adipocytes through a post-transcriptional mechanism via changes in glucose transport. This starvation-like response may be physiologically important in conditions such as uremia to prevent excessive energy expenditure.

Protein-energy malnutrition is a salient feature in humans with chronic renal failure. Metabolic acidosis, a common component of uremia, leads to malnutrition by increasing protein degradation in skeletal muscle (1–3). In contrast, apart from one study showing that low pH directly inhibits lipolysis in isolated rat adipocytes (4), the effect of a low pH on adipose tissue is largely unexplored. Consequently, the role of acidosis in regulating specific adipocyte-secreted proteins and/or genes, especially those implicated in nutrition pathophysiology, is unknown.

The adipocyte hormone leptin communicates the body's nutritional status to regulatory centers in the brain to inhibit appetite, increase energy expenditure, and reduce fat stores (5). Plasma leptin is strongly correlated with fat mass and increases in parallel with adipose stores (6). In contrast, circulating leptin acutely decreases after fasting or calorie restriction in both rodents and humans (7,8). Like starvation, metabolic acidosis is associated with lower serum leptin in uremic rats (9). How-

ever, the mechanism or mechanisms that link acidosis and leptin is unknown.

In uremic patients, serum leptin commonly reaches levels several times higher than would be expected for their adipose mass (10). Leptin is a 16-kD molecule filtered at the glomerulus and extensively metabolized in the renal tubules. Several studies have therefore suggested that serum leptin rises because of a reduced renal clearance (11). However, nearly one-third of patients with end-stage renal disease (ESRD) maintain normal plasma leptin, and uremic hyperleptinemia is not fully explained. Typical factors associated with ESRD, such as chronic inflammation, hyperinsulinemia, and endotoxemia, have all been shown to increase leptin gene expression (11–13). In contrast, Nordfors *et al.* (11) have demonstrated that in patients with advanced chronic renal failure, leptin mRNA levels from adipose tissue appeared to be decreased when compared with healthy subjects. The authors speculated that hyperleptinemia *per se* directly downregulates leptin gene expression. Whether other factors associated with uremia, conceivably acidosis, contribute to low leptin mRNA levels in these patients is unknown.

There is now accumulating evidence that leptin is nutritionally regulated. Studies in isolated rat adipocytes have shown that glucose transport and metabolism are major regulators of leptin protein secretion and gene expression (14). Furthermore, the intracellular flux of glucose into the hexosamine biosynthetic pathway rapidly regulates leptin gene expression in fat and skeletal muscle from rats (15). Because glucose transport

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is a known pH-dependent process, we hypothesized that acidosis leads to a reduction of glucose transport and hence downregulates leptin gene expression and leptin protein secretion.

The purpose of this study was to test this hypothesis *in vitro* by using the 3T3-L1 adipocyte cultured cell model; by determining the effect of low pH on leptin secretion and leptin mRNA; by measuring glucose transport into adipocytes after incubation with acid; and finally by testing whether glucose transport inhibitors affect leptin mRNA and leptin release.

Materials and Methods

DMEM, MEM, FBS, and penicillin-streptomycin and glutamine were purchased from Invitrogen. Insulin, dexamethasone, 3-isobutyl-1-methylxanthine (IBMX), cytochalasin B, phloretin, and 2-deoxy-D-glucose were obtained from Sigma-Aldrich. 2-Deoxy-D-[2,6-³H] glucose was purchased from Amersham. Cell culture plastics were supplied by Nunc.

Cell Culture

3T3-L1 mouse fibroblasts were obtained from the European Collection of Animal Cell Cultures (ECACC; reference 86052701). For leptin secretion studies, cells were plated at 5×10^4 cells per well in 35-mm six-well plates and routinely maintained in 2 ml of DMEM with 10% heat-inactivated FBS, 500 IU/ml penicillin, 500 μ g/ml streptomycin, and 10 mg/L phenol red at 37°C under 5% CO₂. When heavily confluent, differentiation into adipocytes was initiated by the addition of 500 μ M IBMX, 250 nM dexamethasone, and 330 nM insulin to the medium for 2 d. Cells were then further cultured without hormones for another 4 d. The medium was changed three times a week during the propagation phase and every other day during the differentiation phase. Experiments were started 6 d after induction of differentiation. At this stage, phase contrast microscopy showed that all of the cells exhibited typical adipocyte morphology without any apparent fibroblast contamination. For leptin mRNA studies, cells were treated similarly, except that they were plated at an initial density of 12×10^4 cells in 25-cm² flasks with vented caps.

Leptin Protein Quantitation

Leptin concentrations in cell culture media were determined using a sandwich ELISA for mouse leptin (Quantikine M; R&D Systems) as described previously (9).

Analysis of Leptin mRNA

Total RNA was extracted using a monophasic phenol and guanidine isothiocyanate (Trizol reagent; Invitrogen), according to the original manufacturer's instructions. Minor modifications were made to overcome potential interference of high lipid levels with the extraction process. Briefly, after removal of the test medium, the cells were washed three times with PBS and lysed in 1.5 ml Trizol reagent. The lysates were transferred into RNase-free tubes. A total of 300 μ l of chloroform was added to each tube, which was vortexed for 15 s before standing at room temperature for 15 min. The sample was then centrifuged at 11,000 rpm ($11600 \times g$) at 4°C for 15 min. Lipids (on the top) were removed, and the aqueous phase was retained. The RNA was precipitated from the aqueous phase by adding 750 μ l isopropanol, vortexing, and standing for 10 min at room temperature before again centrifuging at 4°C, 13,000 rpm, for 10 min. The RNA pellet was washed in 75% ethanol and resuspended in 40 μ l of diethylpyrocarbonate water. Total RNA was stored at -70°C .

The leptin cDNA probe was synthesized by reverse transcriptase (RT)-PCR from untreated 3T3-L1 adipocyte RNA. First-strand cDNA was synthesized by reverse transcription of 1.6 μ g total RNA using the Reverse Transcription System (Promega) according to manufacturer's instructions. The resulting first-strand cDNA was amplified by PCR using ReddyMix PCR Mastermix (ABgene) and specific primers custom-made by Life Technologies. The primers were designed on the basis of the published sequence of the mouse *ob* gene (16). The sense primer was 5'-ATG TGC TGG AGA CCC CTG TGT CGG-3', and the antisense primer was 5'-GCA TTC AGG GCT AAC ATC CAA CTG-3'. Thermocycling conditions were as follows: denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 68°C for 2 min for a total of 40 cycles (Techne Genius). The resulting 508-bp PCR product was electrophoresed on a 1% agarose Tris-acetate-EDTA (TAE) gel containing ethidium bromide and purified using Sephaglas BandPrep Kit (Pharmacia Biotech) according to the manufacturer's instructions. The sequence of the PCR product (nucleotide position 144 to 652 of the murine *ob* gene sequence) was verified by oligonucleotide sequencing using the ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction System according to manufacturer's instructions.

Northern blot analysis with the above cDNA leptin probe failed to detect leptin mRNA in 3T3-L1 cells. This finding is consistent with the fact that the level of leptin gene expression by 3T3-L1 adipocytes is quite low (approximately 1%) relative to that in mouse adipose tissue (17). Consequently, leptin mRNA was assessed by semiquantitative RT-PCR and Southern blot test. Briefly, 1 μ g of each RNA sample was converted into first-strand cDNA and amplified via PCR as described above. Aliquots of cDNA were electrophoresed on a 1% agarose TAE gel containing ethidium bromide. The resulting cDNA was denatured for 45 min in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) and then neutralized for 45 min in buffer containing 1.5 M NaOH, 0.5 M Tris pH 7.5. Samples were transferred onto Hybond-N nylon membranes (Amersham Pharmacia) by capillary action using 20 \times SSC. Membranes were prehybridized for 4 h at 42°C in a solution containing 50% formamide, 1% SDS, 5 \times Denhardt, and 5 \times SSPE (saline sodium phosphate ethylenediaminetetra-acetic acid) and 200 μ g/ml salmon sperm DNA. The membranes were hybridized overnight with a [³²P] dCTP cDNA probe that had been labeled, using a random primer labeling system (Prime-a-Gene; Promega). After hybridization, the membranes were washed twice with 1% SDS, 2 \times SSPE at room temperature for 10 min, twice with 1% SDS, 0.2 \times SSPE at 65°C, and then exposed to X-Omat LS film (Kodak) with intensifier screens at -70°C . Densitometric analysis of the transcripts was carried out on a BioRad GS 700 imaging scanner (BioRad).

Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as the housekeeping gene to normalize leptin cDNA transcripts for loading. RT products were amplified using specific rat GAPDH primers (R&D Systems). Thermocycling conditions were: denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 45 s for a total of 35 cycles. The resulting PCR products were electrophoresed on a 1% TAE agarose gel containing ethidium bromide. The resulting densitometric signals were quantified as above and used for leptin cDNA transcript normalization.

Assessment of Cell Viability

Cell viability was assessed by measurement of lactate dehydrogenase (LDH) activity released into the cell-culture supernatant using a commercial spectrophotometric assay (Sigma DG1340-K). 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 ANOVA with Duncan's multiple range test. Statistical significance was defined as *P* < 0.05.

Experimental Design

For leptin secretion studies, 3T3-L1 adipocytes were exposed for up to 4 d to test medium at acidic pH 7.1 or control pH 7.5. The pH was adjusted by addition of HCl or NaHCO₃, with addition of NaCl to the acidic cultures to maintain a constant Na concentration. This test medium contained MEM, 10% heat-inactivated FBS with the addition of 500 IU/ml penicillin, and 500 μg/ml streptomycin as above, and was changed after 48 h. Aliquots of media were collected at 48 and 96 h and frozen at -20°C for leptin assays. For leptin mRNA experiments, the cells were treated for 48 h, after which 1.5 ml Trizol was added. The flasks were subsequently stored at -20°C before RNA extraction.

Transport of glucose from extracellular medium into the cells was assayed as described by Estrada *et al.* (18). Briefly, after incubation under the test conditions, cultured adipocytes were rapidly washed twice with HEPES-buffered saline (NaCl 140 mM, HEPES acid 20 mM, MgSO₄·7H₂O 2.5 mM, KCl 5.0 mM, and CaCl₂·2H₂O 1.0 mM, pH 7.4). Cells were subsequently incubated in HEPES-buffered saline

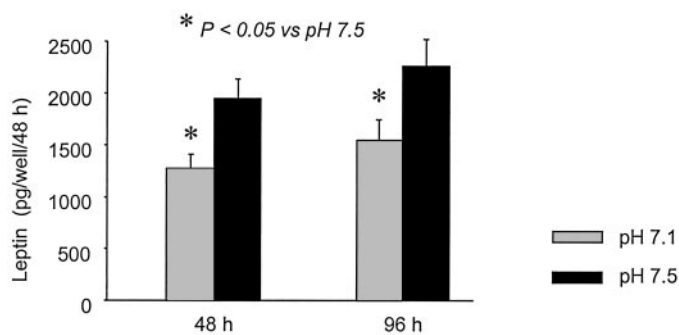


Figure 1. Effect of low pH on leptin secretion in 3T3-L1 adipocytes. 3T3-L1 adipocytes in six-well plates were incubated for 48 or 96 h in pH 7.1 or 7.5. Leptin concentrations in culture supernatants were assayed by ELISA. Results are mean ± SEM of two independent experiments (pool of 21 to 24 culture wells for each set of conditions).

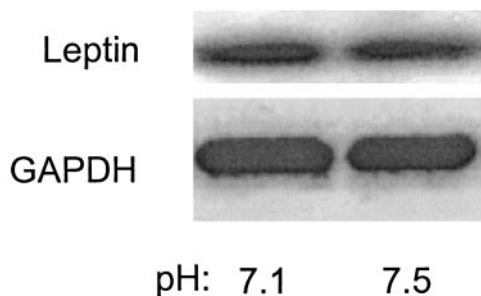


Figure 2. Effect of acidosis on 3T3-L1 adipocyte leptin gene expression. 3T3-L1 adipocytes were exposed to pH 7.1 or 7.5 for 48 h. pH did not affect leptin mRNA as assessed by Southern blot test. Results show a representative blot from three separate experiments. GAPDH, glyceraldehyde phosphate dehydrogenase.

containing 10 μmol/L 2-deoxy-D-[2,6-³H] glucose (1 μCi/ml) for exactly 5 min at room temperature.

Glucose uptake was rapidly terminated by placing the culture plates on ice and aspirating the radioactive incubation medium and washing the cells three times with ice-cold 0.9% NaCl. Nonspecific uptake, determined in parallel incubations by inhibiting transporter-mediated uptake with 10 μmol/L cytochalasin B, was subtracted from all

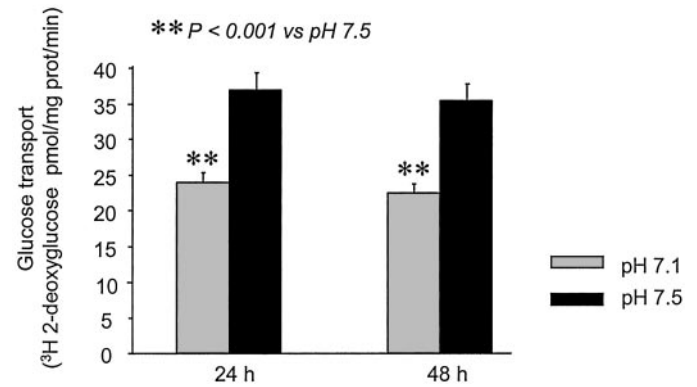


Figure 3. Effect of pH on glucose transport in 3T3-L1 adipocytes. 3T3-L1 adipocytes were exposed to a pH of 7.1 or 7.5. Cells were washed and incubated for 5 min with 2-deoxy-D-[³H]glucose (2-DG). Results are means of nine independent experiments (pool of 33 culture wells for each set of conditions).

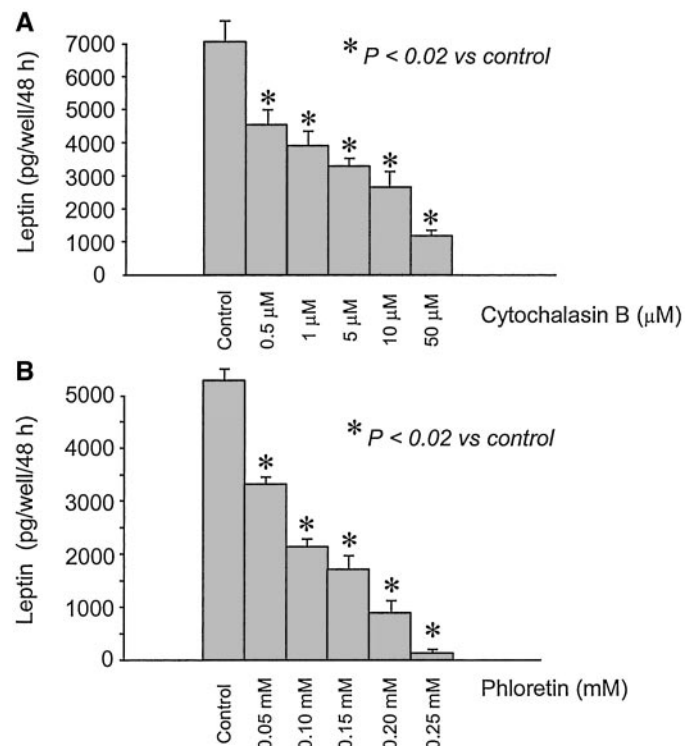


Figure 4. Effect of cytochalasin B and phloretin on leptin secretion. 3T3-L1 adipocytes in six-well plates were incubated for 48 h with various concentrations of the glucose transport inhibitors cytochalasin B (A) and phloretin (B). Leptin was assayed in the cell supernatants. Data are expressed as pg/well/48 h and are means of two separate experiments (pool of 10 to 12 wells for each set of conditions).

measurements. Cells were lysed in 0.05 M NaOH for 30 min at 70°C, and the lysate radioactivity was determined by liquid scintillation counting (Ecoscint A, National Diagnostics).

Adipocytes were exposed to varying concentrations of glucose transport inhibitors (*i.e.*, cytochalasin B and phloretin) in MEM containing 10% FBS for 48 h. The media were collected and stored at –20°C before assay for leptin protein. Total DNA, protein, and triglycerides were extracted from the cells exactly as described previously (9). For leptin mRNA experiments, the cells were treated similarly, after which 1.5 ml Trizol was added. The flasks were subsequently frozen at –20°C before RNA extraction.

Results

Effect of Acidosis on Leptin Secretion and Leptin Gene Expression

A low pH of 7.1 was associated with a significantly reduced leptin output both at 48 and 96 h, confirming our previous report (9) (Figure 1). In contrast, leptin mRNA was not affected by low pH at 48 h (Figure 2).

Effects of Acidosis on Glucose Transport

Because low pH did not affect leptin gene expression, acidosis was thought to act directly on leptin release through changes in nutrient supply, especially glucose transport. At pH 7.1, glucose uptake was indeed significantly reduced at 24 and 48 h (Figure 3).

Effects of Glucose Transport Inhibitors on Leptin Secretion and Leptin mRNA

To determine whether inhibiting glucose transport by methods other than acidification was able to reduce leptin secretion,

3T3-L1 adipocytes were incubated with the glucose transport inhibitors cytochalasin B and phloretin. Both agents significantly reduced leptin secretion in a dose-dependent fashion at 48 h ($P < 0.02$ versus control; Figure 4). As shown in Table 1, total intracellular protein, DNA, and triglycerides were unaffected by cytochalasin B and phloretin up to 0.15 mM. Therefore, the reduction in leptin secretion by glucose transport inhibition was not due to changes in cell size, number, or stage of adipocyte differentiation. Moreover, this effect was not due to cytotoxicity because LDH release was only significant at the highest doses of phloretin (0.20 and 0.25 mM).

Cytochalasin B at low doses (0.5 and 1 μ M) did not affect leptin mRNA, although these concentrations reduced leptin secretion by 36% and 43%, respectively, as shown above. In contrast, cytochalasin B at higher doses (5 and 10 μ M) and phloretin at 0.05 and 0.10 mM suppressed leptin mRNA (Figure 5).

Dose-Response Relationship between Glucose Transport Inhibitors and Glucose Uptake

Acidosis decreased both glucose transport and leptin secretion by approximately 40% (Figures 1 and 3). Dose-response curves for the inhibition of glucose transport by cytochalasin B and phloretin (Figure 6) showed that cytochalasin B at 0.5 and 1.0 μ M reduced glucose transport to a level comparable with that observed with acid. These doses of cytochalasin B also readily suppressed leptin secretion (Figure 4) without affecting leptin mRNA (Figure 5A), confirming that this level of glucose transport inhibition could account for the acid-induced decrease in leptin secretion, independent of changes in leptin

Table 1. Effect of cytochalasin B and phloretin on total DNA, protein, and intracellular triacylglycerol (triglyceride) levels in 3T3-L1 adipocytes^a

Transport Inhibitor	DNA (μ g/well)	Protein (μ g/well)	Triglyceride (mmol/L)	Percentage LDH Release
Cytochalasin B				
control	38.5 \pm 1.2	736 \pm 12	1.13 \pm 0.21	0.19 \pm 0.03
0.5 μ M	35.8 \pm 2.1	721 \pm 14	1.28 \pm 0.31	0.15 \pm 0.02
1 μ M	34.8 \pm 1.4	715 \pm 23	1.27 \pm 0.26	0.16 \pm 0.02
5 μ M	33.2 \pm 2.5	696 \pm 31	1.05 \pm 0.15	0.27 \pm 0.03
10 μ M	34.1 \pm 2.8	698 \pm 22	1.12 \pm 0.22	0.30 \pm 0.04
50 μ M	32.9 \pm 2.3	666 \pm 24	1.08 \pm 0.17	0.82 \pm 0.10
Phloretin				
control	40.1 \pm 1.9	803 \pm 18	1.27 \pm 0.31	0.27 \pm 0.05
0.05 mM	40.1 \pm 2.1	794 \pm 24	1.42 \pm 0.36	0.25 \pm 0.04
0.10 mM	38.6 \pm 1.4	795 \pm 28	1.02 \pm 0.23	0.33 \pm 0.05
0.15 mM	38.7 \pm 2.8	790 \pm 41	1.31 \pm 0.25	0.39 \pm 0.03
0.20 mM	33.2 \pm 1.8 ^b	635 \pm 42 ^b	1.11 \pm 0.32	5.30 \pm 0.74 ^b
0.25 mM	28.5 \pm 2.2 ^b	592 \pm 73 ^b	1.18 \pm 0.19	10.82 \pm 0.90 ^b

LDH, lactate dehydrogenase.

^a 3T3-L1 adipocytes in six-well plates were incubated for 48 h with various concentrations of the glucose transport inhibitors cytochalasin B and phloretin. Total intracellular DNA, protein and triglycerides were assayed according to previously published methods (9). LDH activity in culture supernatants is expressed as a percentage of total cell LDH activity. Data are pooled from three separate experiments and are mean \pm SEM of 13 to 19 culture wells for each set of conditions.

^b $P < 0.05$ versus control.

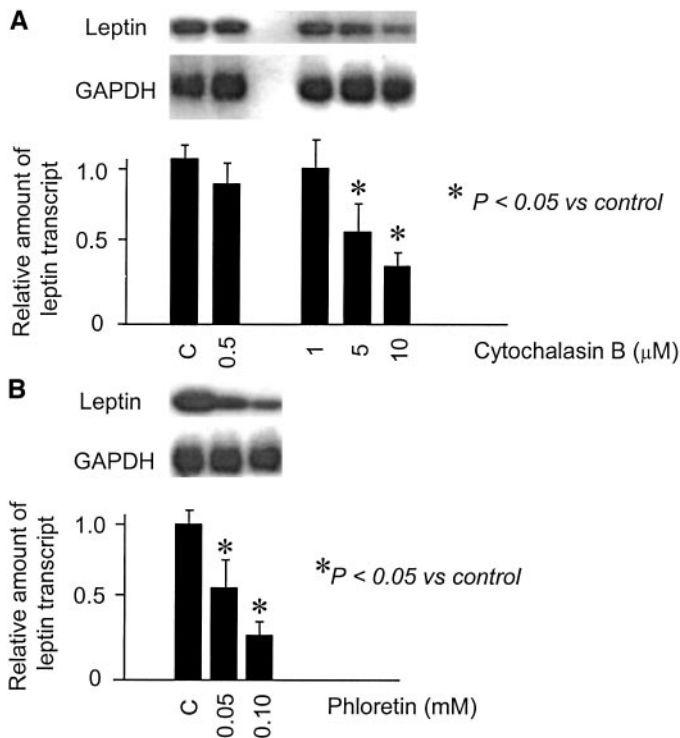


Figure 5. Effects of cytochalasin B and phloretin on leptin gene expression. 3T3-L1 adipocytes in 25-cm² flasks were incubated for 48 h with various concentrations of cytochalasin B (A) and phloretin (B). Leptin mRNA was assessed by Southern blot test. Results show a representative blot from three separate experiments. GAPDH, glyceraldehyde phosphate dehydrogenase.

mRNA. In contrast, higher doses of cytochalasin B induced a dramatic blockade in glucose uptake (more than 80%), which in turn was sufficient to decrease leptin mRNA. Furthermore, phloretin (0.05 to 0.15 mM) induced a greater suppression of glucose uptake (83% to 90%) than acidosis, and this was also capable of downregulating leptin mRNA, which as a result led to a greater reduction in leptin secretion than acid.

Discussion

Our data show that a low pH of 7.1 decreased glucose transport in 3T3-L1 adipocytes, a finding consistent with earlier work that showed that glucose transport was increased in response to a raised pH in other cell types, including rat adipocytes (19,20). This decrease in glucose transport was comparable in magnitude to the decrease in leptin secretion in response to low pH. Furthermore, this effect was apparent at 24 h, preceding the effect on leptin secretion, which occurred at 48 h. To test the hypothesis that the effect on leptin is driven by the changes in glucose transport, we used glucose transport inhibitors. As expected, leptin release progressively decreased with increasing concentrations of cytochalasin B or phloretin. The results are consistent with those obtained by Mueller *et al.* (14), who demonstrated a similar effect of glucose transport blockade on leptin release in isolated rat adipocytes.

To our knowledge, no previous study has investigated the effect of acidosis on leptin mRNA. The major finding of this

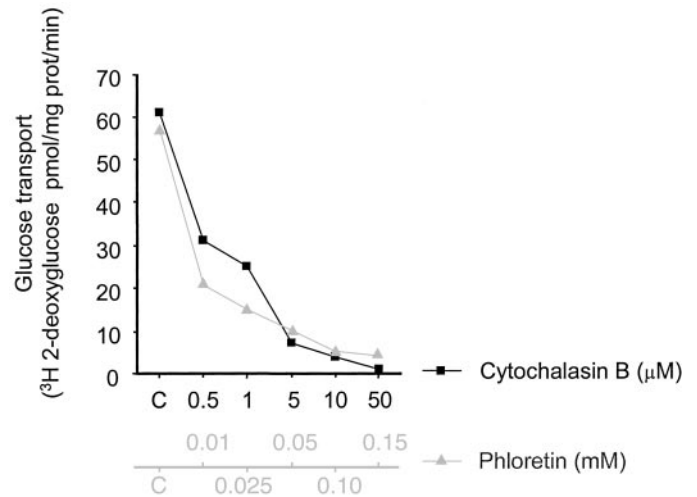


Figure 6. Relationship between glucose transport inhibitors and glucose uptake. 3T3-L1 adipocytes were exposed for 24 h to various concentrations of glucose transport inhibitors. The cells were washed and incubated in 2-deoxy-D-[³H]glucose (2-DG) for 5 min. Radioactivity in cell lysates was determined with a β-scintillation counter. Data were obtained from three separate experiments. Each point shown on the graph is a mean of 10 to 12 culture wells for each set of conditions.

study was that a low pH did not affect leptin gene expression but reduced leptin secretion. The observed link between glucose transport and leptin output implies that some aspect of intracellular glucose metabolism influences leptin synthesis, turnover, or secretion. Wang *et al.* (15) demonstrated that in rats, increasing the glucose flux into the hexosamine biosynthetic pathway led to increased UDP-*N*-acetylglucosamine (UDP-GlcNac), the end product of this pathway, which in turn induced a rapid increase in leptin mRNA and protein levels. Similarly, Considine *et al.* (21) showed that treatment of human subcutaneous adipocytes with glucosamine, an intermediate in UDP-GlcNac biosynthesis, increased leptin release, although leptin mRNA was not measured in those experiments.

However, in the study presented here, leptin secretion from 3T3-L1 adipocytes was shown to be decreased, independent of leptin mRNA content. Therefore, although it is well established that changes in glucose metabolism via the hexosamine pathway can induce changes in leptin secretion at transcriptional level, our data suggest that glucose transport also exerts an additional post-transcriptional effect on leptin output. In our study, leptin gene expression was in fact only affected when glucose transport was dramatically suppressed (at least 80%), which is much greater than the acid-induced glucose transport inhibition. Such dissociation between effects on leptin mRNA and leptin release has been described elsewhere in response to conditions other than low pH (22). This observation supports the notion that leptin secretion may occur from a preexisting intracellular pool, independent of *de novo* synthesis (23).

The inhibition of glucose transport and leptin secretion by low pH bears an interesting resemblance to changes observed during starvation (24). The fasting-induced fall in leptin secre-

tion is believed to serve as an important afferent signal to the brain aimed to trigger adaptive responses to maintain energy balance during starvation (7). Because metabolic (keto)acidosis is a well documented feature of starvation, this effect may reinforce the action of other starvation signals, such as hypoinsulinemia, in reducing glucose transport and leptin secretion.

Similarly to starvation, uremia is a life-threatening condition in humans. In this situation, hyperleptinemia may add a further potential threat to survival through increasing energy expenditure and decreasing appetite. From these results, we hypothesize that in uremic patients, acidosis may be sensed by the adipose tissue to rapidly decrease its leptin production as an adaptive response to restore serum leptin to normal. This effect may counteract the uremic hyperleptinemia and therefore prevent the negative energy balance that otherwise might threaten survival. The clinical relevance of these data are supported by the observation that in patients with ESRD, especially those with low adiposity, serum leptin levels may be similar to controls (25). Thus, acidosis may mask uremic hyperleptinemia in some patients. When acidosis is corrected by the administration of sodium bicarbonate, serum leptin significantly increases (26), which is further consistent with our data.

In summary, this study provides evidence that glucose transport contributes to the inhibition of leptin secretion by acid *in vitro*, independently of leptin mRNA changes; thus, the effect of a low pH on glucose transport is to modulate leptin output by a post-transcriptional mechanism.

Acknowledgments

This study is dedicated to the memory of John Walls, whose untimely death occurred during the course of this work and deprived us of his leadership. Professor Walls brought invaluable contributions to the current knowledge on the effects of metabolic acidosis in renal disease. Part of this work was presented at the International Congress on Nutrition and Metabolism in Renal Disease (Lyon, France, July 6 to 8, 2000) and at the European Dialysis and Transplant Association Congress (Nice, France, September 17 to 20, 2000). D.T. was a research fellow supported by the Swiss National Science Foundation and by grants-in-aid from the Fondation du 450e Anniversaire de l'Université de Lausanne and the Fondation SICPA (Lausanne, Switzerland) and the Stiftung zur Förderung der Ernährungsforschung in der Schweiz (Zürich, Switzerland).

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